STUDIES AND APPLICATIONS INVOLVING

MICROBIOLOGICAL ASSAYS

Approved:

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STUDIES AND APPLICATIONS INVOLVING MICROBIOLOGICAL ASSAYS

DISSERTATION

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The University of Texas in Partial Fulfillment
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By

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CHAPTER I

INTRODUCTION

(49) applied Lactobacillus casei to the assay of arginine, glutamic acid, leucine, phenylalanine, tryptophan, tyrosine, and valine. A few months later, McMahan and Snell (37) also reported the use of L. casei in the determination of arginine and valine. With these early methods as a pattern, methods for the other amino acids were rapidly developed and today it is possible to use microbiological methods for the assay of at least 18 amino acids.

The reliability of a particular method and the validity of assay values obtained by such a method can be checked in several ways. An assay method is usually considered reliable if the analytical results obtained do not show a trend to either higher or lower results as the amount of sample is increased. If there is a drift toward lower results as the amount of sample is increased it is possible that an inhibitor is present in the sample. Evidence of a stimulatory material in the sample or limiting medium conditions are indicated when assay values increase as the concentration of sample is increased. A method is also considered reliable when assay values can be reproduced from day to day or when the values obtained check the values obtained by some other established method. More faith can also be placed in analytical results when the same answer is obtained with two or more microorganisms.

In addition to these criteria of reliability of assays methods there are several other factors to be considered in the development of microbiological procedures. One of these, which was a serious limitation in the early stages of the development of the microbiological amino acid methods, is the purity of the commercial amino acids used in the basal media. In

(49) applied Lactobacillus casei to the assay of arginine, glutamic acid, leucine, phenylalanine, tryptophan, tyrosine, and valine. A few months later, McMahan and Snell (37) also reported the use of L. casei in the determination of arginine and valine. With these early methods as a pattern, methods for the other amino acids were rapidly developed and today it is possible to use microbiological methods for the assay of at least 18 amino acids.

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In addition to these criteria of reliability of assay methods there are several other factors to be considered in the development of microbiological procedures. One of these, which was a serious limitation in the early stages of the development of the microbiological amino acid methods, is the purity of the commercial amino acids used in the basal media. In

many instances it is impossible to demonstrate the essential nature of a given amino acid for a microorganism because of the presence of this amino acid as a contaminant in the other amino acids used in the basal medium. As a result of this fact reports on the essential nature of certain amino acids for different microorganisms have not been consistent.

Another factor to be considered is the configuration of standards, L or DL, used in the assays. In some instances the D form is inactive, which permits the use of a synthetic DL standard. In other cases, however, the D form is either inhibitory or stimulatory necessitating the use of a standard which matches the unknown. Other important factors which affect the accuracy of an assay will be discussed in due course.

Botanical classification places the Chinese tallow tree, Sapium sebiferum, in the Euphorbiaceae or Spurge family (2). It grows to heights of greater than forty feet with a branch spread of approximately the same dimension. Because of its rapid growth and beautiful autumn foliage, the tree has been used to some extent as a ornamental plant. The white tallow coated nuts produced by this tree first appear as green pods. These pods, each containing three nuts, occur in clusters at the end of small branches. As the nuts mature, the pod cover dries and falls off, leaving the white tallow coated nuts exposed.

It is this small nut, approximately one-fourth of an inch in diameter, that has given potential importance to the Chinese tallow tree. The chief interest in the tallow nut has been centered around the high quality drying oil that can be obtained from the nut. Both the oil (23, 25, 43, 45) and the tallow (21, 24, 40, 43) have been rather thoroughly investigated as to properties and methods of processing. However, the protein residue which remains

after the removal of oil, tallow, and hull has not been investigated.

Chapters II and III present the results of the investigation of the B-vitamin and amino acid content of two protein preparations secured in the processing of the Chinese tallow nut. Chapters IV, V, and VI give the results of investigations of special problems which arose during the amino acid analyses.

CHAPTER II

B-VITAMIN CONTENT OF CHINESE TALLOW NUT PREPARATIONS

B-VITAMIN CONTENT OF CHINESE TALLOW NUT PREPARATIONS

B-Vitamin Assay Methods. Lactobacillus casei was used as the assay organism for biotin, folic acid, niacin, pantothenic acid, and riboflavin. The medium used for the assays was that described by Roberts and Snell (46) and is given in Table I. Thiamin was determined by the method of Sarett and Cheldelin (48) which uses Lactobacillus fermentum 36 as the assay organism. Medium conditions for thiamin assay are given in Table I. Saccharomyces carlsbergensis 4228 was employed for pyridoxine assays and Saccharomyces cerevisiae for inositol determinations. The medium of Atkin, Schultz, Williams and Frey (1), Table I, was used for both pyridoxine and inositol.

Assay Samples. Two Chinese tallow nut preparations were assayed for their B-vitamin content. One of these was a high protein content flour which contained some hull and fiber. The other sample was a purified protein prepared from the flour. The flour was prepared from nuts freed of twigs and branches by screening and hand picking. The tallow was removed from the nuts by counter current extraction with hexane in a pilot plant extractor described by Harris (19). The nuts were then cracked in a Bauer mill. The kernels were successfully removed from the majority of the hulls by flotation in ethylene dichloride. In this solvent the kernels floated and the hulls sank. The kernels were thus separated from the hulls and were easily removed by merely dipping them off by means of a wire gauze. The kernel fraction was freed of oil by extraction with ethylene dichloride in a large Soxlet type extractor, and then air dried and ground in a small laboratory grinding mill. This preparation contained 10.11 per cent moisture, 6.45 per cent ash, and 10.86 per cent nitrogen.

TABLE I

COMPOSITION OF COMPLETE BASAL MEDIA FOR VITAMIN DETERMINATIONS

Constituent	For Biotin, Niacin, Riboflavin, Pantothenic Acid and Folic Acid Assays	For Inositol and Pyridoxine Assays	For Thiamin Assays
	Amount per 10 ml. final medium		
	ml.	ml.	mg.
Acid hydrolized casein	---	0.5	25
Enzymatic casein digest	4.0	---	---
Potassium citrate buffer	---	0.5	---
	mg.	mg.	
Alkali treated peptone	---	---	100
Sodium acetate	200	---	60
Cystine	1.0	---	1.0
$CaCl_2 \cdot 2H_2O$	---	1.25	---
$FeCl_3$	---	0.025	---
$FeSO_4 \cdot 7H_2O$	0.4	---	0.1
KCl	---	4.25	---
KH_2PO_4	25	5.5	5
K_2HPO_4	25	---	5
$MgSO_4 \cdot 7H_2O$	8	1.25	2
$MnSO_4 \cdot 4H_2O$	1.6	0.037	0.1
NaCl	0.4	---	0.1
Adenine sulfate	0.1	---	0.1
Guanine hydrochloride	0.1	---	0.1
Uracil	0.1	---	0.1

TABLE I (continued)

COMPOSITION OF COMPLETE BASAL MEDIA FOR VITAMIN DETERMINATIONS

Constituent	For Biotin, Niacin, Riboflavin, Pantothenic Acid and Folic Acid Assays	For Inositol and Pyridoxine Assays	For Thiamin Assays
	Amount per 10 ml. final medium		
	γ	γ	γ
Biotin	0.04	0.08	0.004
Calcium pantothenate	5	25	1
Folic acid[1]	0.02	---	0.0025
Inositol	---	250	---
Nicotinic acid	5	---	1
p-Aminobenzoic acid	1	---	1
Pyridoxine	10	5	1
Riboflavin	5	---	1
Thiamin	5	2.5	---

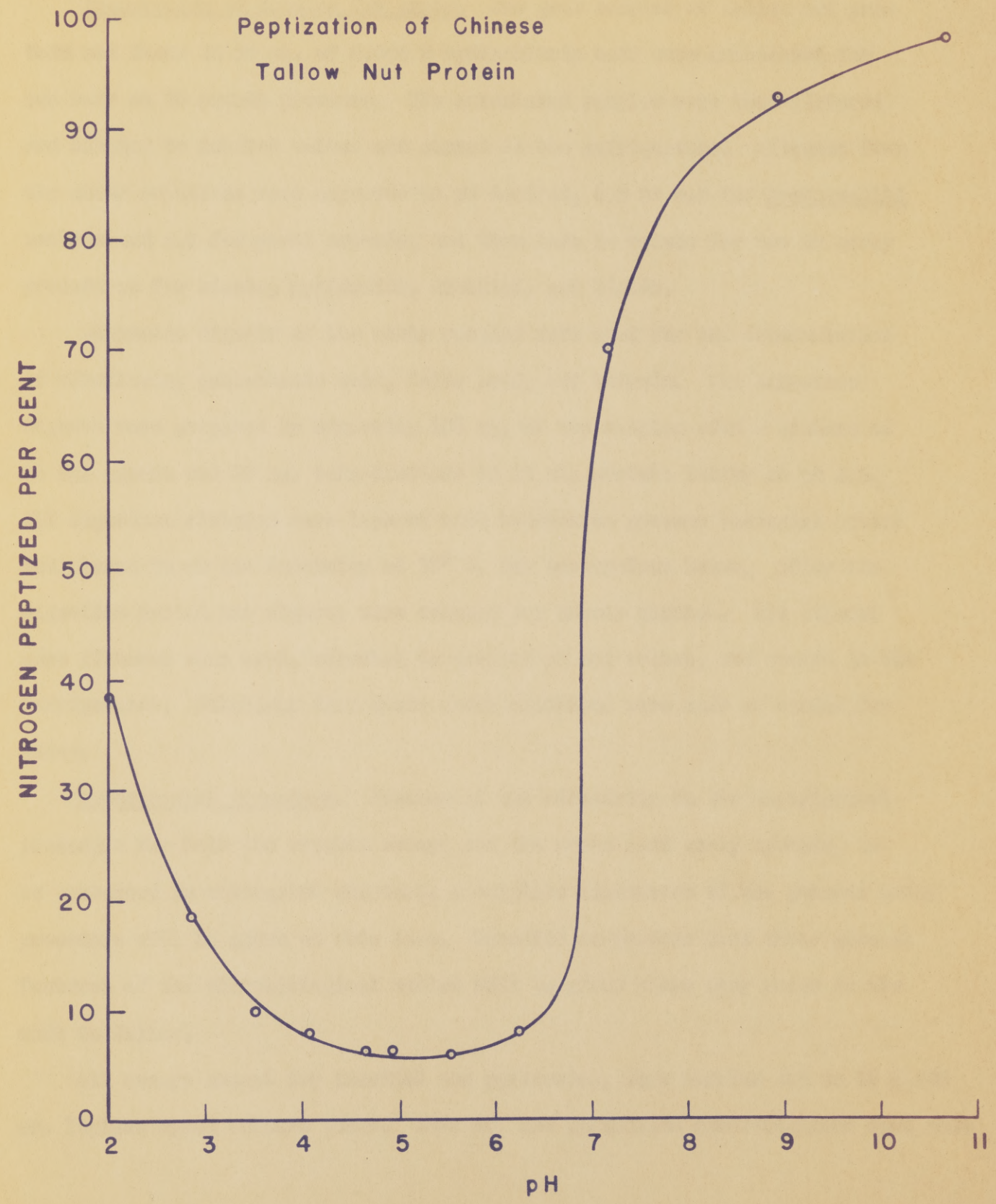
[1]The name folic acid, whenever used in this dissertation, means pteroylglutamic acid. This material was obtained through the courtesy of Lederle Laboratories Division, American Cyanamid Company.

In the preparation of the protein from the tallow nut flour, it was first necessary to determine the solubility characteristics of the protein with reference to the pH values of the solution. For this study the method of Painter and Nesbitt (41) was employed. The method consists of adding 3 grams of the nut flour to 100 ml. volumes of hydrochloric acid and sodium hydroxide solutions of varying hydrogen ion concentration. The solutions were shaken for 2 hours and then centrifuged. An aliquot of the supernatant was taken for nitrogen analysis by the micro-Kjeldahl method of Ma and Zuazaga (34). Another aliquot of the supernatant was used for pH determination. Results of the peptization studies are given in Figure 1.

As shown by Figure 1, the nitrogen present in the flour has a minimum solubility at pH 5.5 with a marked increase in solubility at pH 6.5 to 7.0. At pH 8.5 over 90 per cent of the nitrogen is in solution. With the pH solubility relationship determined the protein was prepared by dissolving 3 parts of the tallow nut flour in 100 parts of water at 25° C. and pH 8.5. The insoluble material was then removed by filtration and the filtrate adjusted to pH 5.5 with hydrochloric acid. This change in pH caused the protein to precipitate. The protein was allowed to settle and the excess supernatant liquid removed by decantation. The remainder of the liquid was removed from the protein precipitate by centrifugation. The precipitate was washed with 3 portions of acetone and then dried under vacuum. The protein obtained was a fluffy white material containing 8.75 per cent moisture, 1.08 per cent ash, and 15.96 per cent nitrogen.

FIGURE 1

Peptization of Chinese Tallow Nut Protein



Preparation of Samples for Assay. One gram samples of tallow nut protein and flour in 50 ml. of 0.055 N hydrochloric acid were autoclaved for one hour at 20 pounds pressure. The autoclaved samples were then filtered and diluted to desired volume and stored in the refrigerator. Aliquots from the stock solutions were adjusted to pH desired, 6.5 to 6.8 for Lactobacilli methods and 5.2 for yeast methods, and then made to volume for use in assay procedures for niacin, pyridoxine, inositol, and biotin.

Enzymatic digests of the assay samples were used for the determination of riboflavin, pantothenic acid, folic acid, and thiamin. The enzymatic digests were prepared by digesting 100 mg. of the samples with a mixture of 20 mg. papain and 20 mg. taka-diastase in 10 ml. acetate buffer at pH 4.5. All digestion mixtures were layered with benzene to prevent bacterial action and placed in an air incubator at 37° C. for twenty-four hours. After the digestion period the digests were steamed for twenty minutes. The digests were filtered when cool, adjusted to desired pH and volume, and stored in the refrigerator. Dilutions from these stock solutions were made as needed for assay.

Experimental Procedure. Because of the similarity in the experimental procedure for both the vitamin assays and the amino acid assay methods, to be presented in subsequent chapters, a complete discussion of the general assay procedure will be given at this time. Specific deviations from these general features of the microbiological method will be given where they occur in the work to follow.

All assays except for inositol and pyridoxine, were carried out in 18 x 150 mm. lipless Pyrex culture tubes. Inositol and pyridoxine determinations were made

in 50 ml. Erlenmeyer flasks. To prepare the tubes or flasks for assay, graded amounts of sample and standard vitamin or amino acid solutions were added to the culture tubes. The tubes were then diluted to a volume of 5 ml. with distilled water. Five ml. of appropriate double strength medium were then added. The appropriate medium contained all known metabolites essential for growth except the one to be determined. These metabolites essential for growth were all supplied in excess so that the possible addition of metabolites as sample would have no effect on the growth response to the limiting metabolite being determined. The tubes thus made to final volume of 10 ml. were either plugged with non-absorbent cotton or capped with aluminum caps and sterilized by autoclaving for 10 minutes at 15 pounds pressure. For thiamin, inositol, and pyridoxine, sterilization with flowing steam in the autoclave at atmospheric pressure was employed.

The sterilized tubes were then cooled and inoculated with one drop of suspension of lactic acid bacteria called for in the methods employed. Inocula were prepared from 18 to 24 hour old cultures grown at 37° C. The liquid cultures were centrifuged and the supernatant decanted. The cells were then resuspended in 10 ml. of sterile 0.9 per cent saline solution and centrifuged. This washing with saline was repeated and the washed cells were suspended in 100 ml. of saline and one drop, approximately 0.05 ml., of the resulting suspension was used to inoculate the assay tubes. After inoculation, the tubes were placed in a water bath at 35° C. and incubated for 16 to 24 hours for turbidimetric growth measurements and three to four days for titrimetric growth measurements.

The amount of growth in thiamin, inositol, and pyridoxine assays was measured by the turbidimetric method. For these measurements a Coleman Junior Spectrophotometer was used. An uninoculated tube containing 5 ml. of basal medium and 5 ml. of distilled water, was used to adjust the instrument to 100 per cent transmission. Bacterial or yeast growth was measured as a function of the turbidity produced. The extent of turbidity was reflected in the galvanometer readings of the spectrophotometer, the greater the turbidity the lower the reading. The extent of growth in assays for biotin, niacin, riboflavin, folic acid, and pantothenic acid was measured by titrating with 0.1 N sodium hydroxide the lactic acid produced by the lactic acid bacteria. All titrations were made with the aid of a MacBeth continuous indicating pH meter. The large calomel and glass electrodes were inserted into a titration vessel made from the bottom half of a 500 ml. globe separatory funnel. The outer surface of the titration vessel was shielded with aluminum foil and grounded to the calomel electrode. The air driven stirrer and stirrer bearing were both made of plastic because a metal stirrer and bearing caused fluctuations in the pH meter. Glass was also eliminated because it has a tendency to become brittle at the bearing surface and as a result could break more easily. This possibility of breakage would endanger the unprotected electrodes. The sodium hydroxide burette was also installed above the titration cup and was equipped with automatic filling and leveling devices. The assay tubes were titrated to a given pH, usually that of an uninoculated tube which ranged from pH 6.6 to 7.0.

Inositol and pyridoxine assays were made in 50 ml. Erlenmeyer flasks. Due to the size of yeast cells it is necessary to shake the yeast flasks to

prevent the yeast cells from settling to the bottom of the flask. In such a situation, with the cells in the bottom of the flask, the diffusion of the metabolites to the cells would be a factor which could vary from tube to tube or flask to flask. However, by the use of flasks instead of culture tubes adequate shaking was obtained to prevent the cells from settling out. Shaking was supplied by a horizontally oscillating shaker.

In assays with yeast it is also necessary to standardize the size of the inoculum. For this standardization the turbidity of yeast cell suspensions of known weight of moist yeast per unit volume were measured on the Coleman spectrophotometer. A curve of galvanometer reading against weight of moist yeast cells was constructed from the data. This curve was then used to determine the weight of yeast cells present per unit volume of inoculum. With the concentration known, appropriate dilution with sterile saline solution gave the desired strength of inoculum. One-tenth milligram of moist yeast cells was used per flask for the estimation of both inositol and pyridoxine.

The growths obtained in the standard tubes containing varying amounts of a pure vitamin or amino acid were plotted against the concentration of the vitamin or amino acid. Growths, expressed either in ml. 0.1 N sodium hydroxide or per cent transmission, were plotted as the ordinate and concentration of metabolite as the abscissa. These growth curves thus represented the quantitative response of the microorganism over a range of metabolite concentration. The growths obtained from known weights of sample hydrolysates were located on the standard curve for a particular assay and the concentrations of vitamin or amino acid corresponding to these growths were taken from the abscissa. These concentrations divided by the weight of sample gave the concentration of the assay metabolite per unit weight of sample, usually expressed as micrograms (γ) per gram.

Significance of B-Vitamin Assay Values. The B-vitamin values for the Chinese tallow nut products presented in Table II have little significance unless compared to other products of established usage. To make possible such a comparison, B-vitamin assay values for whole wheat flour and dried brewer's yeast are also given in Table II. These two products represent natural materials which serve to increase the vitamin content of the finished product into which they are incorporated. Thus whole wheat flour added to white flour increases the vitamin content of bread or other bakery products. Dried brewer's yeast, because of its high B-vitamin content, is used extensively as a vitamin supplement in animal feeds and to a limited extent as a vitamin supplement for humans.

The Chinese tallow nut flour, because of the hulls present, could not be used to enrich white flour unless the hulls were first removed by screening and bolting. However, the protein preparation could be used for such a purpose. A comparison of the vitamin values of the tallow nut protein and whole wheat flour in Table II shows that the former would furnish greater quantities of thiamin and pyridoxine per unit weight than the latter, but would be less adequate in enrichment of pantothenic acid and niacin. On a unit weight basis neither the Chinese tallow nut flour nor the protein could compare to dried brewer's yeast as a relatively complete vitamin supplement to animal feed or as a source of vitamins for human consumption.

Another approach to ascertain the adequacy of the vitamins in the tallow nut preparations is to consider the amount of vitamins that would be added if these materials were used as sources of protein in the diet of animals. Thus in preparing a 20 per cent protein diet with tallow nut flour as the protein

TABLE II

B-VITAMIN CONTENT OF CHINESE TALLOW NUT FLOUR AND PROTEIN

Vitamin	Chinese Tallow Nut		Whole Wheat Flour (8)	Brewer's Yeast (58)
	Flour	Protein		
	γ/g.	γ/g.	γ/g.	γ/g.
Biotin	0.29	0.04	0.058	0.071
Folic acid	0.60	0.37	2.0[2]	1.05[2]
Inositol	750	225	1890	280
Niacin	29.8	9.9	46.0	126.0
Pantothenic acid	2.4	0.41	13.0	42.5
Pyridoxine	17.3	13.9	2.2	1.0
Thiamin	100.7	80.8	5.6	8.5
Riboflavin	3.6	0.74	1.8	15.2

[2]Micrograms of material of "potency" 40000.

source, the amount of each vitamin added, expressed in micrograms per 100 grams of ration, would be as follows: biotin-9.3, niacin-950, pantothenic acid-90, pyridoxine-550, thiamin-3200, and riboflavin-115. These values gain further significance when compared to amounts of vitamins added to diets of different experimental animals. For example, Richardson and Hogan (44) add the following amounts of vitamins, γ per 100 grams, to their experimental rat diet: biotin-10, niacin-5000, pantothenic acid-920, pyridoxine-400, thiamin-400, and riboflavin-400. If these amounts are compared to those that would be supplied by the tallow nut flour to give a 20 per cent protein diet it is apparent that pantothenic acid, riboflavin, and niacin would be deficient. The deficiency in niacin would not be a factor in the diet of the rat because this vitamin is not required. However, the amounts of riboflavin and pantothenic acid would limit the growth of the rat.

According to the Committee of Animal Nutrition of the National Research Council starting chicks should be fed a diet containing 20 per cent protein and B-vitamins in micrograms per 100 grams of ration as follows: biotin-10, niacin-1750, pantothenic acid-1100, pyridoxine-350, thiamin-200, and riboflavin-350. Comparing these vitamin concentrations with those that would be supplied by the tallow nut flour at a level to supply 20 per cent protein, it is apparent that the flour would supply only sufficient pyridoxine and thiamin. Biotin would also possibly be adequate. However, the small amounts of pantothenic acid, riboflavin, and niacin furnished by the tallow nut flour would necessitate the addition of these vitamins from other sources.

The comparisons that have been made in discussing the vitamin content of the tallow nut preparations have all been based on using these materials

as the only source of vitamins in the feed of the rat and the chick. This in general is not the case because the rations usually contain other vitamin containing materials such as wheat bran, yeast and alfalfa leaf meal. However,the comparisons given suffice to show the limitations in the B-vitamins and to show the supplementary value of the tallow nut flour and protein with respect to thiamin and pyridoxine.

CHAPTER III

AMINO ACID CONTENT OF CHINESE TALLOW NUT PROTEIN AND FLOUR

AMINO ACID CONTENT OF CHINESE TALLOW NUT PROTEIN AND FLOUR

Amino Acid Assay Methods. The methods used for the determination of the amino acids, Table III, were selected primarily on their established value for such assays and secondarily on the number of amino acids that could be assayed for by one organism and basal medium condition. The second factor, when it could be applied, saved time in preparation of media and thus facilitated the routine determinations of a larger number of amino acids. For many of the amino acid determinations, the same organism was used with different media conditions and in other assays two or more organisms were used in assay procedures. This procedure was adopted in order to eliminate possible variations in response to different media and to eliminate variations in response by different organisms.

Assay Samples. The Chinese tallow nut flour and protein described in Chapter II were used as assay samples for the amino acid determinations.

Preparation of Samples for Assay. One gram samples of the flour and protein were hydrolized by boiling with 100 ml. of constant boiling hydrochloric acid (1:1) for 24 hours. After the hydrolysis period, the hydrolysate was reduced to a thick syrup by removing the hydrochloric acid and water by distillation on a steam bath under reduced pressure. The syrup was dissolved in distilled water and the mixture again reduced to a thick syrup by vacuum distillation. The residue was again taken up in distilled water and filtered, adjusted to pH 6.8 and diluted to contain 10 mg. of the sample per ml. Hydrolysates were stored in the refrigerator and dilutions made from these stock solutions as needed for assay purposes.

TABLE III

MICROORGANISMS AND ASSAY METHODS USED FOR AMINO ACID ASSAYS

Amino Acid	Microorganism	Medium
Alanine	S. faecalis R	Lyman, Moseley, Wood, Butler and Hale (33)
Arginine	S. faecalis R	Guirard, Snell and Williams (15) Stokes, Gunness, Dwyer and Caswell (54)
Aspartic acid	Leuc. mesenteroides P-60	Hac and Snell (16)
Cystine	Leuc. mesenteroides P-60	Medium A, Meinke and Holland (38)
Glutamic acid	L. arabinosus 17-5	Hac, Snell and Williams (17) Lyman, Kuiken, Elotter and Hale (29)
Glycine	Leuc. mesenteroides P-60	Medium A, Meinke and Holland (38)
Histidine	S. faecalis R Leuc. mesenteroides P-60	Stokes, Gunness, Dwyer and Caswell (54) Guirard, Snell and Williams (15)
Isoleucine	L. arabinosus 17-5 S. faecalis R	Kuiken, Norman, Lyman and Hale (28) Stokes, Gunness, Dwyer and Caswell (54)
Leucine	L. arabinosus 17-5 S. faecalis R	Kuiken, Norman, Lyman and Hale (28) Stokes, Gunness, Dwyer and Caswell (54)
Lysine	S. faecalis R Leuc. mesenteroides P-60	Stokes, Gunness, Dwyer and Caswell (54) Guirard, Snell and Williams (15)
Methionine	Leuc. mesenteroides P-60 S. faecalis R	Guirard, Snell and Williams (15) Stokes, Gunness, Dwyer and Caswell (54) Lyman, Moseley, Wood and Hale (32)

TABLE III (continued)

MICROORGANISMS AND ASSAY METHODS USED FOR AMINO ACID ASSAYS

Amino Acid	Microorganisms	Medium
Phenylalanine	L. arabinosus 17-5	Kuiken, Norman, Lyman and Hale (28)
	L. delbrueckii 5	Stokes, Gunness, Dwyer and Caswell (54)
Proline	Leuc. mesenteroides P-60	Guirard, Snell and Williams (15)
		Dunn, Shankman, Camien, Frankl and Rockland (10)
Serine	S. faecalis R	Baumgarten, Mather and Stone (3)
	Leuc. mesenteroides P-60	Meinke and Holland (38)
	L. delbrueckii 5	Stokes, Gunness, Dwyer and Caswell (54)
	L. casei	McMahan and Snell (37)
Threonine	S. faecalis R	Guirard, Snell and Williams (15)
		Stokes, Gunness, Dwyer and Caswell (54)
Tryptophan	L. arabinosus 17-5	Lyman, Moseley, Wood and Hale (32)
	S. faecalis R	
Tyrosine	L. casei	Lyman, Moseley, Wood and Hale (32)
	Leuc. mesenteroides P-60	
Valine	L. arabinosus 17-5	Guirard, Snell and Williams (15)
	S. faecalis R	Stokes, Gunness, Dwyer and Caswell (54)
		Kuiken, Norman, Lyman and Hale (28)

Experimental Procedure. The general microbiological assay technique described in Chapter II was also used for the amino acid determinations. Amino acids used as standards were those of Merck and Co. or General Biochemicals, Inc. With the exception of one lot of isoleucine and several lots of alanine, which were contaminated with leucine and glycine, respectively, all the individual amino acid preparations from these two sources were free of other amino acids.

Results of Amino Acid Determinations. Results of the amino acid determinations are given in Table IV. The results are presented as average assay values for each amino acid and as a range or variation in values obtained. The figures given in the column marked "Range" of Table IV represent the high and low average values obtained on different hydrolysates or checks on the same hydrolysate run at different times. Using the average assay values of the amino acids as a basis of comparison for both the flour and the protein, the range or variation of seven of the amino acids, arginine, aspartic acid, cystine, glycine, leucine, threonine, and valine, was less than 5 per cent. A deviation from the average of 7 per cent or less but greater than 5 per cent was obtained with alanine, glutamic acid, isoleucine, methionine, phenylalanine, serine, and tryptophan. The range of assay values for the other four amino acids, histidine, lysine, proline, and tyrosine, differed from the average reported by as much as 6.3 to 15.4 per cent too low, to 6.3 to 18.8 per cent too high. The average deviation for all the amino acid assays was approximately $\pm$ 4 per cent.

The tallow nut flour and protein, ash and moisture free, contained 13.00 and 17.70 per cent nitrogen. These values corrected for amide nitrogen, 0.73

TABLE IV

AMINO ACID CONTENT OF CHINESE TALLOW NUT PROTEIN AND FLOUR

Amino Acid	Flour		Protein	
	Range	Average	Range	Average
	Per cent	Per cent	Per cent	Per cent
Alanine	1.18 - 1.22	1.20 (2)[1]	1.81 - 2.07	1.94 (2)
Arginine	9.6 - 10.2	10.0 (9)	14.6 - 15.4	15.0 (3)
Aspartic acid	6.7 - 7.0	6.9 (3)	10.1 - 10.8	10.6 (3)
Cystine	0.64 - 0.65	0.65 (3)	1.15	1.15 (2)
Glutamic acid	10.3 - 11.1	10.8 (6)	15.1 - 16.6	15.6 (4)
Glycine	2.9 - 3.0	3.0 (2)	4.3 - 4.6	4.4 (2)
Histidine	1.5 - 1.8	1.6 (10)	2.2 - 2.9	2.6 (7)
Isoleucine	3.3 - 3.6	3.5 (4)	5.2 - 5.5	5.3 (4)
Leucine	4.2 - 4.4	4.3 (6)	6.5 - 6.8	6.7 (4)
Lysine	1.5 - 1.9	1.6 (9)	2.0 - 2.4	2.3 (9)
Methionine	0.89 - 1.01	0.95 (9)	1.40 - 1.49	1.45 (5)
Phenylalanine	2.6 - 3.0	2.8 (8)	4.0 - 4.5	4.3 (8)
Proline	2.5 - 2.9	2.7 (7)	3.6 - 4.1	3.9 (7)
Serine	2.9 - 3.4	3.2 (16)	4.7 - 5.0	4.8 (9)
Threonine	2.1 - 2.3	2.2 (10)	3.3 - 3.4	3.3 (10)
Tryptophan	0.81 - 0.90	0.85(12)	1.21 - 1.33	1.28(16)
Tyrosine	1.8 - 2.1	2.0 (4)	2.9 - 3.5	3.3 (4)
Valine	4.4 - 4.7	4.5 (9)	6.8 - 7.1	7.0 (4)

[1]Figures in parentheses indicate the number of assays.

per cent for the flour and 1.10 per cent for the protein, give amino acid nitrogen values of 12.27 per cent for the flour and 16.60 per cent for the protein. Table V shows the amount of amino acid nitrogen supplied by each amino acid both in the flour and the protein preparation. The amino acid nitrogen recoveries of 11.42 per cent and 15.98 per cent, based on the individual amino acid assays, represent a 93 per cent recovery of the amino acid nitrogen of the original flour preparation and a 96 per cent recovery of the amino acid nitrogen of the protein sample. These recoveries would be better if the losses of amino acids during acid or alkaline hydrolysis could be determined. For example, serine losses during hydrolysis often amount to as much as 10 per cent. By increasing the serine values obtained for the flour and protein by 10 per cent, the amino acid nitrogen recovery would be increased to 94 per cent for the flour and to approximately 96.5 per cent for the protein. Thus in amino acid assays, the actual values may be better than the amino acid nitrogen recovery indicates because of the destruction of some of the amino acids by the acid or alkaline hydrolyses methods employed.

The ratio of the protein amino acid nitrogen to the flour amino acid nitrogen, given in Table V, shows that the method employed for preparing the protein from the flour did not result in a preferential separation of the amino acid nitrogen. That is, in general the ratios given check the average of 1.43 to ± 0.1. This indicates that the protein preparation obtained is representative of the protein present in the original tallow nut.

TABLE V

NITROGEN RECOVERY AS CALCULATED FROM AMINO ACID ASSAYS OF CHINESE TALLOW NUT FLOUR AND PROTEIN

	Amino Acid[2]		Amino Acid Nitrogen		Ratio of Protein to Flour Amino Acid Nitrogen
	Flour	Protein	Flour	Protein	
	Per cent		Per cent		
Alanine	1.5	2.2	0.236	0.346	1.47
Arginine	12.0	16.6	3.860	5.350	1.38
Aspartic acid	8.3	11.7	0.875	1.233	1.41
Cystine	0.8	1.3	0.093	0.152	1.63
Glutamic acid	13.0	17.3	1.238	1.645	1.33
Glycine	3.6	4.9	0.672	0.913	1.36
Histidine	1.9	2.9	0.515	0.785	1.52
Isoleucine	4.2	5.9	0.449	0.630	1.40
Leucine	5.2	7.4	0.550	0.790	1.44
Lysine	1.9	2.6	0.364	0.499	1.37
Methionine	1.1	1.6	0.103	0.150	1.46
Phenylalanine	3.4	4.8	0.289	0.408	1.41
Proline	3.2	4.3	0.390	0.524	1.34
Serine	3.8	5.3	0.508	0.708	1.39
Threonine	2.6	3.7	0.306	0.436	1.42
Tryptophan	1.0	1.4	0.137	0.193	1.41
Tyrosine	2.4	3.7	0.186	0.286	1.54
Valine	5.4	7.8	0.645	0.932	1.44
	Totals:		Totals:		Average:
	75.3	105.4	11.42	15.98	1.43

[2]Amino acid values on ash and moisture free basis. Chinese tallow nut flour - 13.00 per cent nitrogen and Chinese tallow nut protein - 17.70 per cent nitrogen on ash and moisture free basis.

Significance of Amino Acid Content of Tallow Nut Protein. The nutritive value of a protein depends primarily upon its amino acid content, both quantitatively and qualitatively. Zein and gelatin are nutritionally inadequate because of the lack of tryptophan. Also, zein is deficient in lysine; and gelatin is deficient in valine. From the values obtained for the amino acid content of the Chinese tallow nut protein, it would seem to be limiting in alanine, cystine, histidine, lysine, methionine, and tryptophan. However, this conclusion is based only on the low values of these amino acids when compared to the higher values for the others. Such a comparison is of little significance because the requirements are different from one amino acid to the next.

The amino acid composition of egg albumin along with that of the tallow nut protein is given in Table VI. Egg albumin is considered as an adequate protein for animal nutrition when fed at a 20 per cent level in the diet of animals. Thus, taking egg albumin at a 20 per cent level as a standard, the Chinese tallow nut protein at a 20 per cent level would be limiting in alanine, cystine, lysine, and methionine. Of this group the last two are classed as essential for animal nutrition; therefore, the deficiency in alanine and cystine would not be a factor for consideration. In actual feeding tests with rats (39), it was found that a diet containing 10 per cent tallow nut protein supplemented with 0.3 per cent methionine and 0.3 per cent lysine gave growths equal to or slightly better than a diet containing 10 per cent protein from egg albumin. Growth on 10 per cent tallow nut protein diet when unsupplemented or supplemented only with lysine or methionine gave very poor growths. However, the growth may not have been normal on the 10 per cent tallow nut

TABLE VI

COMPARISON OF AMINO ACID CONTENT OF EGG ALBUMIN WITH THE CHINESE TALLOW NUT PROTEIN

	Amino Acid Composition	
	Egg Albumin (4)	Nut Protein
	Per cent	Per cent
Alanine	7.4	2.2
Arginine	5.8	16.6
Aspartic acid	8.2	11.7
Cystine	2.3	1.3
Glutamic acid	16.3	17.3
Glycine	3.3	4.9
Histidine	2.4	2.9
Isoleucine	5.6	5.9
Leucine	9.4	7.4
Lysine	5.1	2.6
Methionine	5.0	1.6
Phenylalanine	5.6	4.8
Proline	4.3	4.3
Serine	7.6	5.3
Threonine	4.5 (22)	3.7
Tryptophan	1.3	1.4
Tyrosine	4.4	3.7
Valine	6.8	7.8

protein supplemented with lysine and methionine because only arginine, isoleucine, and valine would be supplied in sufficient amounts to conform to the minimum requirements as determined by Rose and compiled by Griffith and Farris (13). To meet the minimum amino acid requirements for normal growth of the rat, the diet would have to contain 15 per cent tallow nut protein supplemented with approximately 0.4 per cent methionine and 0.6 per cent lysine. In order to supply sufficient minimum amounts of all the essential acids, the diet would contain approximately 40 per cent tallow nut protein.

The essential amino acids have been considered only from the standpoint of the nutrition of the rat. However, the non-essential amino acids may be of significance under certain conditions. Cystine has a sparing action on methionine and tyrosine can partially replace phenylalanine. However, the non-essential amino acids gain further importance in animal nutrition when the inhibitions caused by some of this group are considered. Growth inhibitions with chickens on diets containing corn or corn products have been attributed to the presence of high quantities of certain amino acids (14), both essential and non-essential. Thus, in any determination of amino acids content of protein materials it is of importance to ascertain the content of both the essential amino acids and the non-essential amino acids.

CHAPTER IV

THREONINE-SERINE ANTAGONISM IN SOME LACTIC ACID BACTERIA

THREONINE-SERINE ANTAGONISM IN SOME LACTIC ACID BACTERIA

Experimental Results. In the course of the determination of the serine content of the Chinese tallow nut protein and flour, it was noted that the standard serine curves obtained with L. delbrueckii, S. faecalis R, Leuc. mesenteroides P-60 and L. casei were sigmoidal in shape. That is, at low concentrations of serine there was a variable lag section followed by a rapid rise in the curve as the serine concentration was increased. At still higher levels of serine the curve fell off as top growth was approached. Figure 2 is representative of the type of standard serine curves with each of the four organisms given above.

The lag section in the standard curves indicated that either serine was not the primary metabolite in serine utilization or there was something present in the medium that was inhibitory to serine utilization. The work of Lyman and associates (29) showed that the lag section in standard curves for glutamic acid could be eliminated by the addition of glutamine. They concluded that glutamic acid was first converted to glutamine before utilization by the organism and that glutamine was the primary metabolite. This work on glutamic acid suggested the use of some compounds that could be derived from serine as means of overcoming the lag in the serine curves. Ethanol amine, a decarboxylation product of serine, and choline, which could arise from the methylation of ethanol amine, were tested. These two compounds had no effect on the lag in the serine curves.

The failure of ethanol amine and choline to alleviate the lag in the serine curve caused a further consideration of the inhibitory property of some constituent in the basal media. A literature survey suggested that the

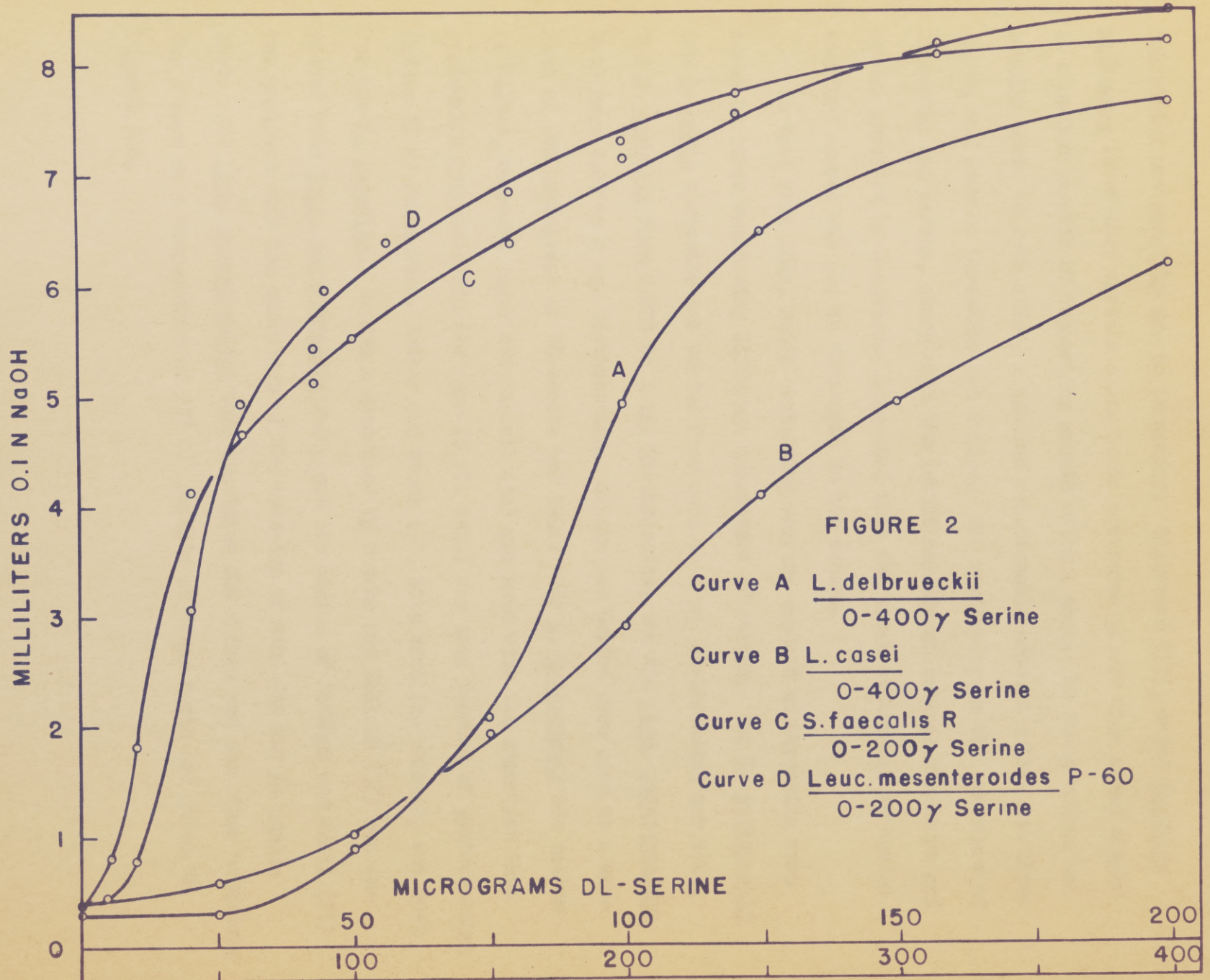


FIGURE 2

Curve A L. delbrueckii
0-400γ Serine

Curve B L. casei
0-400γ Serine

Curve C S. faecalis R
0-200γ Serine

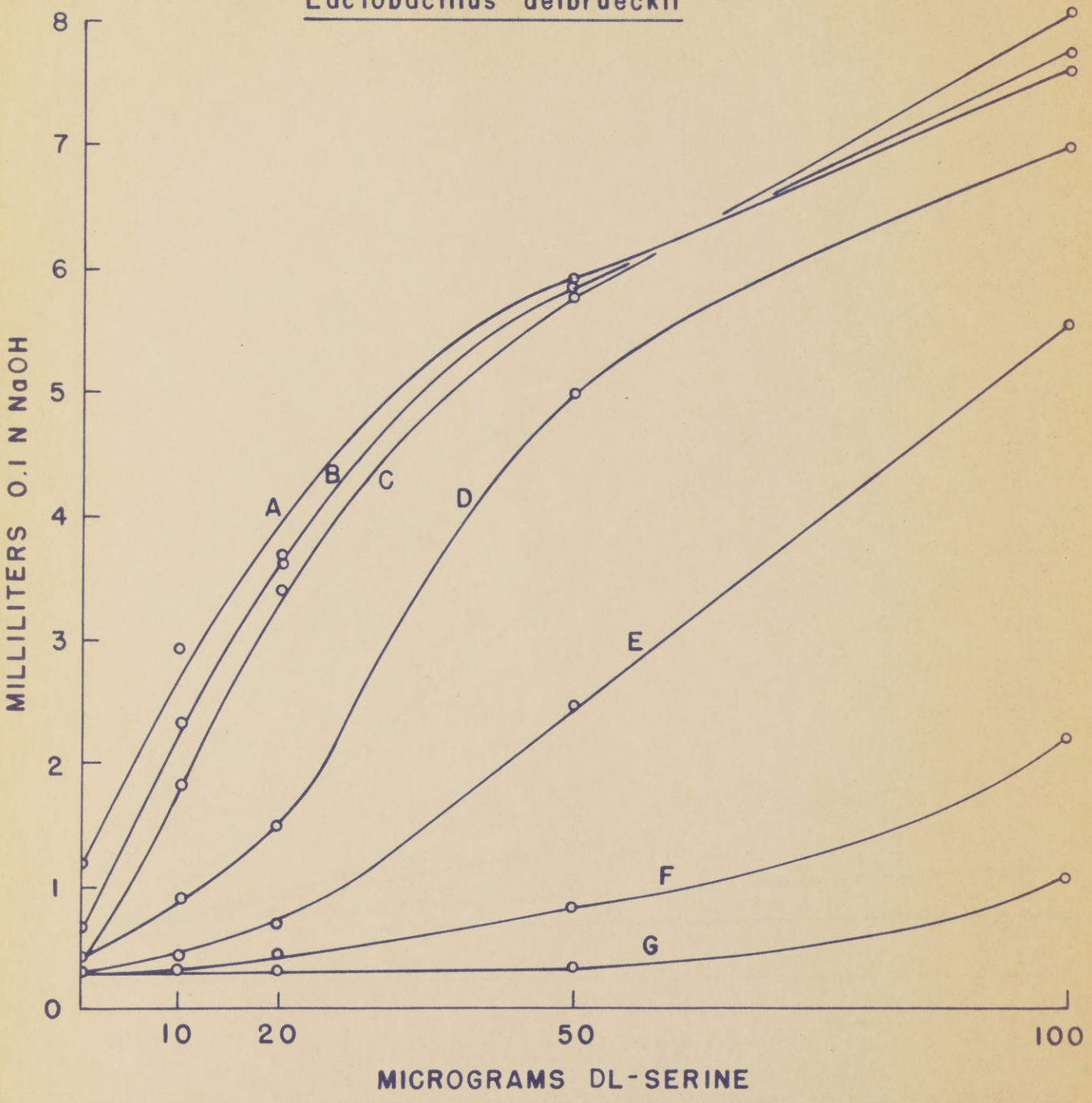
Curve D Leuc. mesenteroides P-60
0-200γ Serine

inhibition was possibly due to threonine. Gladstone (11), using Bacillus anthracis found that certain amino acids prevented growth when added singly to a medium capable of supporting growth without them. He found valine and leucine toxic to each other, a similar relationship between valine and threonine, and between threonine and serine. The similarity in the structure of threonine and serine, along with the inhibitory effect between threonine and serine reported by Gladstone, suggested that the cause of the lag in serine standard curves was due to threonine in the medium.

To test this idea, media containing varying amounts of threonine were added to tubes containing different quantities of serine. For L. delbrueckii and L. casei threonine was varied from zero to 4 mg. DL-threonine per tube, for S. faecalis from 0.025 to 4 mg. DL-threonine, and for Leuc. mesenteroides P-60 from 0.05 to 4 mg. DL-threonine. Serine was varied from zero to a maximum of 100 micrograms of DL-serine per tube. For L. delbrueckii the medium of Stokes, Gunness, Dwyer and Caswell (54) was used with the exception that pyridoxine hydrochloride (20 γ per 10 ml. tube) was used instead of pyridoxamine. Medium II of Baumgarten, Mather and Stone (3), with xanthine omitted, was used for the S. faecalis. Medium A described by Meinke and Holland (38) was employed with Leuc. mesenteroides P-60, and the medium of McMahan and Snell (37) was employed with the exception of the omission of xanthine for L. casei. Tests with Leuc. mesenteroides were incubated for 4 days and the other organisms for 3 days at a temperature of 35° C. Results of these tests are given in Figures 3-6.

FIGURE 3

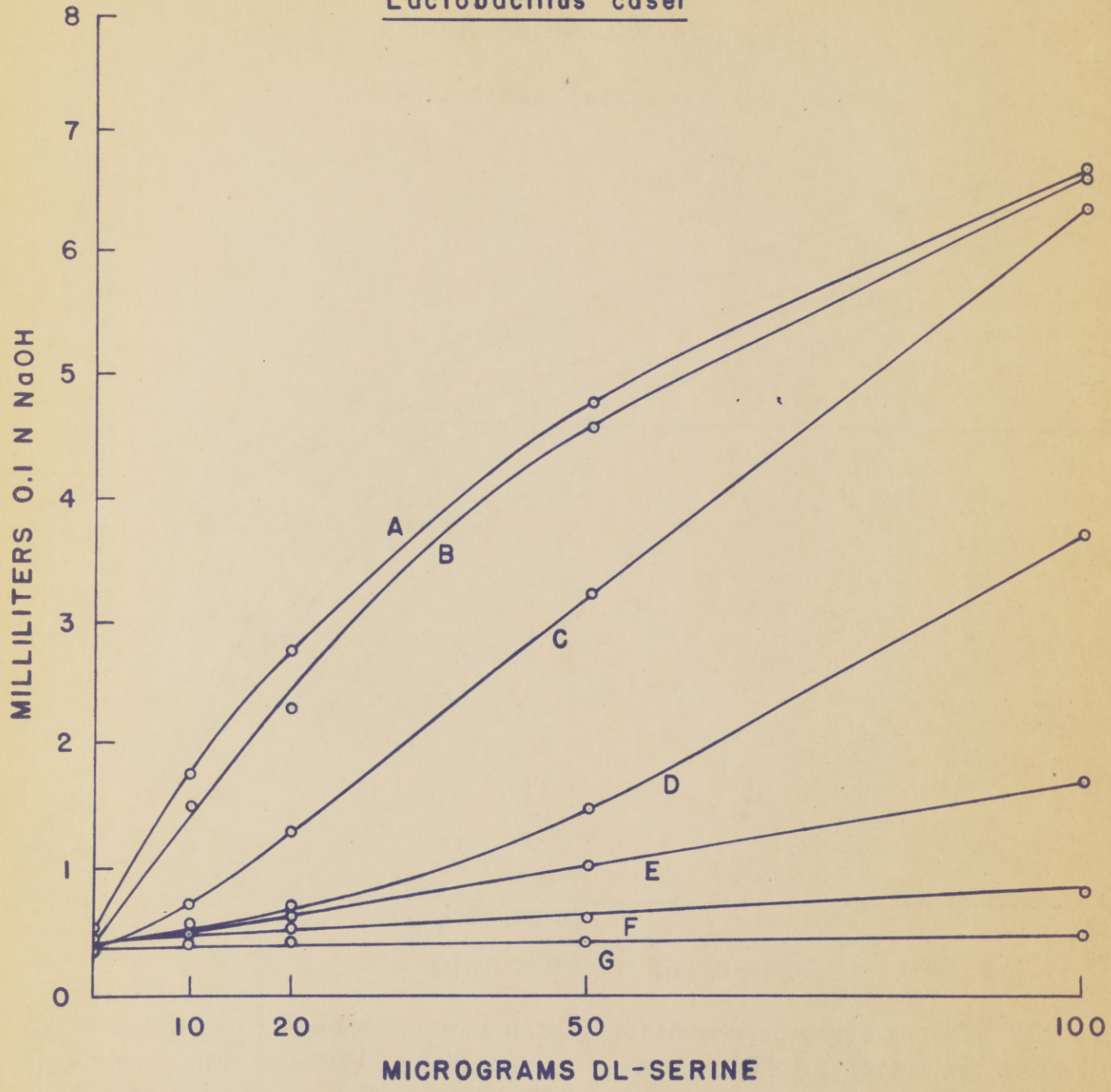
Standard Serine Curves
with
Lactobacillus delbrueckii



Curve A No threonine, Curve B 100γ DL-threonine, Curve C 200γ DL-threonine, Curve D 500γ DL-threonine, Curve E 1000γ DL-threonine, Curve F 2000γ DL-theonine, Curve G 4000γ DL-threonine

FIGURE 4

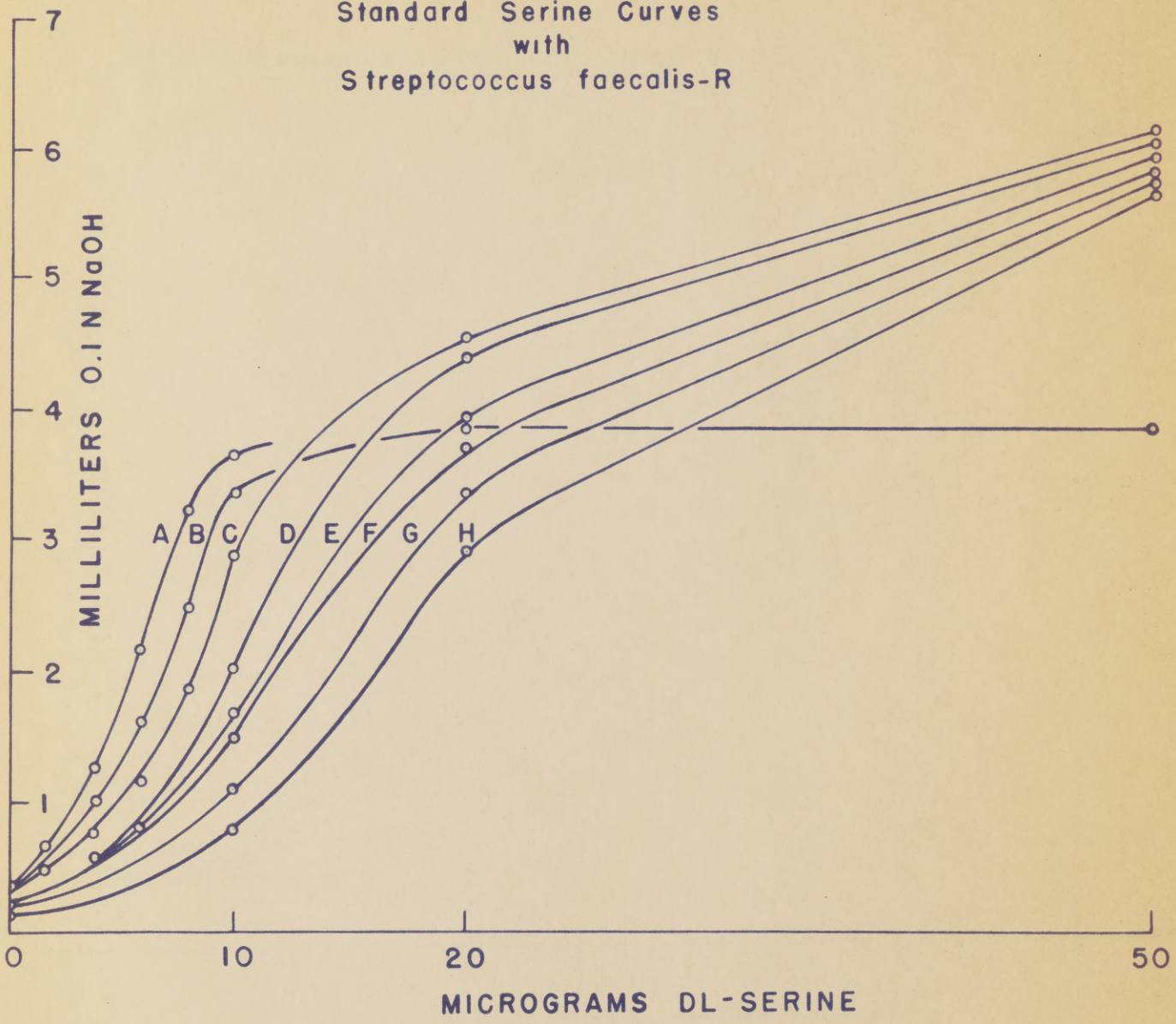
Standard Serine Curves
with
Lactobacillus casei



Curve A No threonine, Curve B 100 γ DL-threonine, Curve C 200 γ DL-threonine, Curve D 500 γ DL-threonine, Curve E 1000 γ DL-threonine, Curve F 2000 γ DL-threonine, Curve G 4000 γ DL-threonine.

FIGURE 5

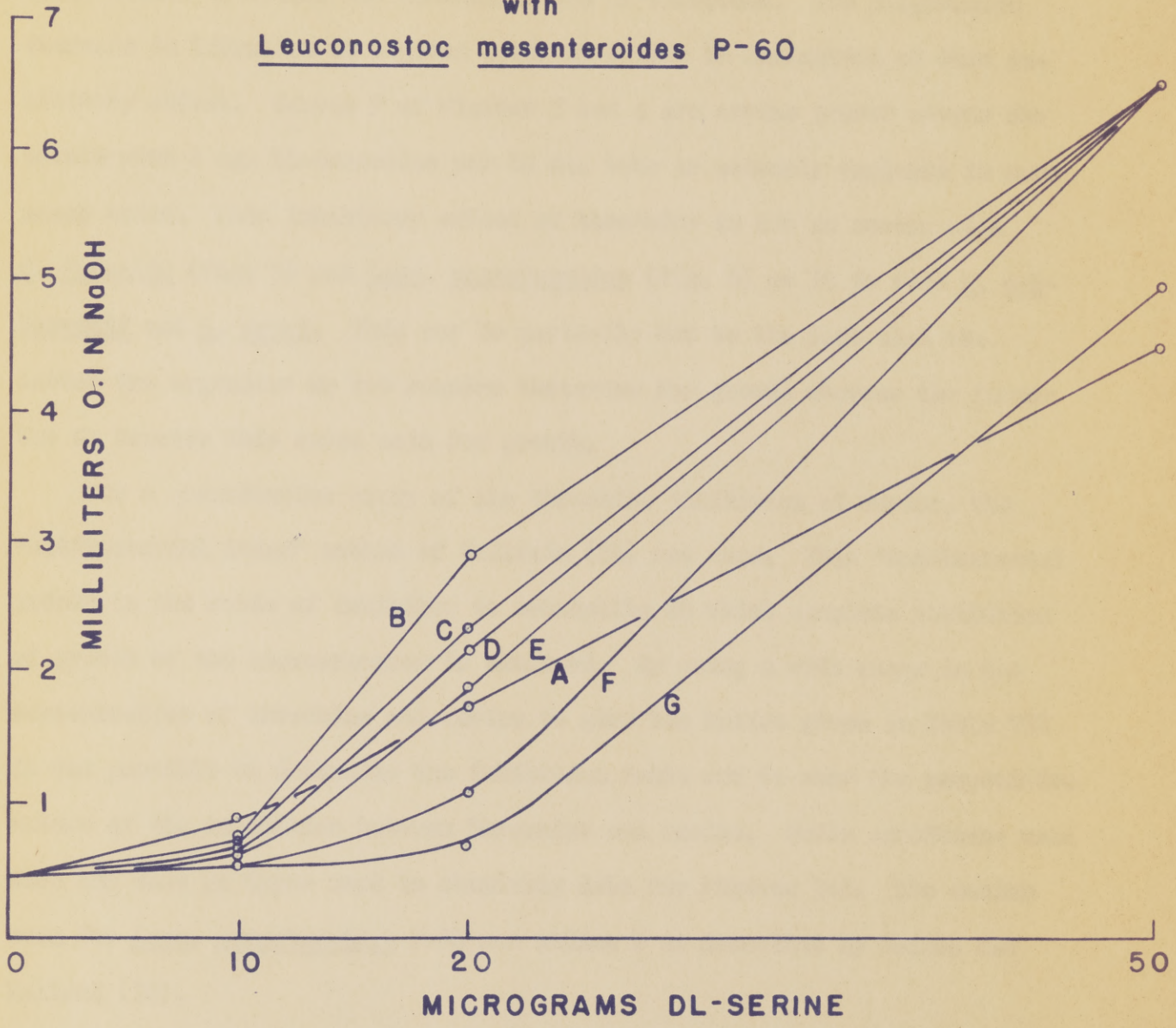
Standard Serine Curves
with
Streptococcus faecalis-R



Curve A 25γ DL-threonine, Curve B 50γ DL-threonine, Curve C 100γ DL-threonine, Curve D 200γ DL-threonine, Curve E 500γ DL-threonine, Curve F 1000γ DL-threonine, Curve G 2000γ DL-threonine, Curve H 4000γ DL-threonine.

FIGURE 6

Standard Serine Curve
with
Leuconostoc mesenteroides P-60



Curve A 50γ DL-threonine, Curve B 100γ DL-threonine, Curve C 200γ DL-threonine, Curve D 500γ DL-threonine, Curve E 1000γ DL-threonine, Curve F 2000γ DL-threonine, Curve G 4000γ DL-threonine.

An analysis of the curves for L. delbrueckii (Fig. 3) and L. casei (Fig. 4) shows that the inhibitory effect of threonine on serine utilization extends to rather low concentrations of threonine. The progressive decrease in titration at a given level of serine is indicative of this inhibitory effect. Curves F of Figures 3 and 4 are serine growth curves obtained with 2 mg. DL-threonine per 10 ml. tube as commonly employed in many assay media. This inhibitory effect of threonine is not as severe with S. faecalis (Fig. 5) and Leuc. mesenteroides (Fig. 6) as it is with L. delbrueckii and L. casei. This may be partially due to the fact that the latter two organisms do not require threonine for growth whereas the former two do require this amino acid for growth.

For a quantitative study of the threonine inhibition of serine, the "antibacterial index" method of McIlwain (36) was used. This "antibacterial index" is the ratio of inhibitor to metabolite at which complete inhibition of growth of the microorganism is obtained. By using a wide range in the concentration of threonine and serine to give the ratios given in Table VII it was possible to determine the inhibition ratio and to show the competitive nature of the inhibition between threonine and serine. Media conditions used were the same as those used in obtaining data for Figures 3-5. The medium used for Leuc. mesenteroides P-60 was medium B as described by Meinke and Holland (38).

Table VII shows the inhibition ratio of threonine to serine to be between 100 and 200 for L. delbrueckii. At a ratio of 100 there was still growth in the tubes which contained 40 mg. threonine. This indicated that there was still serine present in excess of the inhibition exerted by threonine. Quantities of serine left free for growth at a ratio of 100 (in the presence of

TABLE VII

INHIBITION OF SERINE UTILIZATION BY THREONINE

Organism	Threonine	Titration, ml. of 0.05 N NaOH				
		Ratio of threonine to serine				
	mg.	No serine	200	100	50	25
Lactobacillus delbrueckii	0.20	0.70	0.60	0.70	1.05	1.00
	0.50	0.60	0.60	0.60	0.80	0.80
	1.00	0.50	0.55	0.50	0.80	1.36
	2.00	0.52	0.52	0.50	0.74	0.92
	4.00	0.50	0.54	0.55	0.84	1.05
	10.00	0.55	0.60	0.58	1.05	1.50
	20.00	0.56	0.60	0.65	1.20	2.55
	40.00	0.70	0.70	1.20	2.28	13.75
		Ratio of threonine to serine				
	mg.	No serine	100	50	25	10
Lactobacillus casei	0.20	0.85	0.71	0.83	0.91	1.00
	0.50	0.70	0.70	0.82	0.85	1.45
	1.00	0.70	0.70	0.80	0.81	2.30
	2.00	0.70	0.70	0.61	0.80	1.68
	4.00	0.68	0.66	0.71	0.67	2.37
	10.00	0.68	0.68	0.80	0.84	3.60
	20.00	0.70	0.72	0.90	1.13	9.08
		Ratio of threonine to serine				
	mg.	No serine	1000	400	200	100
Leuconostoc mesenteroides P-60	0.50	0.14	0.80	0.94	0.90	0.94
	1.00	0.72	1.00	1.00	0.97	3.78
	2.00	0.80	0.89	0.95	1.33	4.17
	4.00	0.78	0.81	1.15	2.00	8.72
	10.00	0.79	0.90	2.00	7.80	10.80
	20.00	0.85	1.10	3.96	13.10	14.80
		Ratio of threonine to serine				
	mg.	No serine	2000	1500	1000	800
Streptococcus faecalis R	2.00	0.04	0.14	0.34	0.48	0.59
	4.00	0.04	0.34	0.54	0.34	0.62
	10.00	0.00	0.56	0.84	1.32	2.09
	20.00	0.00	0.84	1.51	3.49	4.14
	40.00	0.00	1.84	3.34	7.38	8.84

from 0.2 to 20 mg. threonine) were too small to be significant and as a result blank titrations were obtained. A repeat of the experiment with 100 and 200 micrograms serine, and threonine levels as given in Table VII gave a value of 150 for the "antibacterial index" or inhibition ratio.

Data for L. casei in Table VII indicate an inhibition ratio of between 50 and 100. A repeat of the experiment gave a value of 75. Inhibition ratios of greater than 1000 for Leuc. mesenteroides P-60 and 2000 for S. faecalis R are indicated by titration values given for these two organisms. Best values obtained for the two organisms in later experiments were 1500 for Leuc. mesenteroides P-60 and 2000 to 4000 for S. faecalis R.

If threonine, because of its similarity in structure to serine, can block the utilization of serine, it should be possible for serine to block threonine utilization in organisms requiring threonine. Evidence of such an inhibition is reflected in the data presented in Table VIII. Best values for inhibition ratios of serine to threonine seemto be around 600 for S. faecalis R, 200 for Leuc. mesenteroides P-60, and 400 to 600 for L. arabinosus. These data were obtained with the experimental technique and medium conditions as for Table VII. For L. arabinosus the medium of Lyman et al (28) was used with the omission of the tomatoe eluate and the addition of folic acid.

Discussion of Serine-Threonine Inhibition and Its Influence on Serine Assays.

The technique of heavily dosing a medium with all constituents, other than the one to be assayed, is usually sufficient to eliminate the effect of metabolites added as samples. However, the antagonistic effect of threonine on serine utilization by L. delbrueckii and L. casei, and to a lesser extent with Leuc. mesenteroides and S. faecalis, makes the use of large amounts of threonine

TABLE VIII

INHIBITION OF THREONINE UTILIZATION BY SERINE

Organism	Threonine per tube	Serine per tube, mg.										
	γ	0	0.4	1.0	2.0	4.0	10.0	20.0	40.0	60.0	80.0	100.0
		Titration, ml. of 0.1 N NaOH										
Streptococcus faecalis R	20	0.95	2.86	2.85	2.82	2.74	1.34	0.0				
	100	0.62	7.60	7.42	7.30	7.30	6.30	5.50	4.12	0.78		
	200	0.56	8.62	8.70	8.50	8.52	8.30	8.25	7.70	6.45	4.20	2.00
	20	0.30	0.91	0.30	0.30	0.26	0.32					
Leuconostoc mesenteroides P-60	100	0.32	4.18	4.02		3.85	0.84	0.33	0.32			
	200	0.68	5.80	5.20	4.80	3.68	3.30	3.18	0.30	0.30	0.35	
Lactobacillus arabinosus 17-5	100	10.40			6.35	4.82	2.81	2.04	1.81	1.70	1.72	1.72

in serine assay media undesirable. The use of 2 mg. of DL-threonine per 10 ml. tube, as commonly employed in media, causes a large lag section in the standard serine curves for L. casei and L. delbrueckii. This large lag section in the standard serine curve is objectionable because in the lag section the growth response to added serine is relatively small. This decreases the sensitivity and limits the accuracy of assay values taken from the lag section of the curve. The large lag section also extends the amount of serine or sample that must be added to cover the growth response range of the organism. The addition of more serine is not objectionable but the addition of more assay sample can be a factor.

Media are usually made up containing all known growth factors but this does not preclude the presence of unknown factors in assay samples that may stimulate the growth of the microorganism. Thus the greater the amount of assay sample used, the greater is the possibility of stimulation by unknown constituents of the sample. Conversely, the sample may contain toxic materials which are not significant at low concentrations but may become significant as the concentration of sample per assay tube is increased.

Threonine may be considered as such a toxic material for L. delbrueckii and L. casei. In serine assays with these two microorganisms, the effect of threonine added as sample is an important factor even in the presence of 2 mg. DL-threonine per tube. Curve F (2 mg. DL-threonine per tube) and Curve G (4 mg. DL-threonine per tube) of Figure 3, demonstrate that the addition of threonine in excess of the 2 mg. usually used in assay procedure causes a decrease in titration at a given level of serine. For example, with Curves F and G of Figure 3 there is a difference in titration of 0.5 ml. at 50γ of

serine and 1.2 ml. at 100γ of serine. Experimental data not shown but which represents an extension of these same two curves gave differences in titration of 4.25 ml. at 200γ serine and 3.75 ml. at 300γ serine. These differences were obtained by adding 2 mg. threonine in excess of the amount used in Curve F which represents a large increase in threonine concentration. However, Table IX shows that such an increase in threonine concentration can be approached by the addition of assay samples.

Table IX gives three samples that vary in the ratio of threonine to serine content. Dried cheese whey, with a threonine content approximately three times the serine content, would add a maximum of 950γ L-threonine in the assay range of 0 to 400γ DL-serine (Curve A, Figure 2). This quantity of threonine added as sample approaches very closely the difference in L-threonine content of the media used in obtaining Curves F and G of Figure 3. Such addition of threonine would have a pronounced effect on the response of the organism to serine and as a result would give low assay values which would become increasingly lower as the amount of sample was increased. Silk fibroin on the other hand has a much higher serine content than threonine; therefore, the amount of threonine added would be very small, as is shown by Table IX. The effect of threonine added as sample would be at a minimum. Casein is more representative of most samples on which serine and threonine values have been reported (3, 5). In these samples the ratio of serine to threonine is closer to one. However, data presented in Table IX and Figure 3 show that threonine added as these samples would still exert an effect similar to dried cheese whey but only to a lesser extent.

TABLE IX

COMPARISON OF AMOUNT OF THREONINE ADDED BY SEVERAL SAMPLES IN ASSAY FOR SERINE BY L. DELBRUECKII

Sample	L-Serine	L-Threonine	Assay range, DL-serine	Sample for assay range	L-Threonine added as sample for assay range
	per cent	per cent	γ	mg.	γ
Dried cheese whey	0.04 (3)	1.9 (3)	0-400	0-50	0-950
Casein	6.8 (3)	4.5 (3)	0-400	0-3	0-135
Silk fibroin	11.4 (5)	1.3 (5)	0-400	0-2	0-26
	14.5 (5A)				

The absence of a lag in the L. delbrueckii standard serine Curve A, Figure 3 suggests the use of a medium containing no threonine in the assay of specific samples for serine. As this organism does not require threonine for growth, it would be possible to assay for serine in materials containing no threonine by eliminating threonine from the medium. However, such a method would be very limited and would serve only for samples free of threonine.

In view of the small lag in the standard serine curve for S. faecalis R, it would seem that this organism would be the most desirable for serine assay. However, in the course of the threonine-serine antagonism study it was observed that changes in media conditions had an effect on the serine requirement of this organism. Results of a study of the factors responsible for variations in the serine requirement are presented in the next chapter.

CHAPTER V

INFLUENCE OF THE PURINES AND URACIL, FOLIC ACID, AND CITRATE AND Fe^{++} ON THE SERINE REQUIREMENT OF *STREPTOCOCCUS FAECALIS* R

INFLUENCE OF THE PURINES AND URACIL, FOLIC ACID, AND CITRATE AND Fe^{++} ON SERINE REQUIREMENT OF STREPTOCOCCUS FAECALIS R

In the investigation of the serine-threonine antagonism in some lactic acid bacteria, Medium II of Baumgarten, Mather and Stone (3) was employed for S. faecalis R. Attempts to use other reported S. faecalis R media for serine response studies proved unsatisfactory because it was not possible to obtain blank titrations in the absence of serine. Confusion also existed in the literature as to the essential nature of serine for this microorganism. Serine had been stated as being essential for S. faecalis R by Greenhut, Schweigert and Elvehjem (12), Snell and Guirard (50), and Baumgarten, Mather and Stone (3). However, Dunn, Shankman, Camien and Block (9) found serine to be non-essential for S. faecalis R. These differences suggested that possibly the serine requirement was a function of the media conditions employed.

Evidence of the difference in serine requirement of S. faecalis R under different media conditions is given in Table X. With Medium A serine would be classed as essential, whereas with Media B and C serine would be classed as stimulatory. That is, in the absence of serine under conditions of Medium A there was no growth. Yet, under conditions of Media B and C there was growth in tubes containing no added serine and the addition of serine further stimulated growth.

Purines and Uracil. A comparison of the constituents of the three media used to obtain the data for Table X showed that Medium A contained the highest concentration of the purines and uracil. Medium B contained the smallest

TABLE X

GROWTH OF S. FAECALIS R ON DIFFERENT SERINE-FREE MEDIA

DL-Serine	Medium A (3)	Medium B (31)	Medium C (54)
	Titration, 0.1 N NaOH		
γ	ml.	ml.	ml.
0	0.0	6.43	2.50
4	0.35	6.56	3.01
10	1.90	6.90	3.44
20	4.01	6.63	3.75
30	5.61	6.84	3.80
40	5.92	6.97	3.70
60	6.33	7.33	3.22
80	6.90	7.63	3.17
120	7.41	8.24	3.25
160	7.87	8.70	3.62
200	7.37	8.90	4.15

No xanthine was added to any of the media. Pyridoxine, 16 γ and 20 γ, respectively, was used in Media B and C, instead of pyridoxamine.

concentration. This suggested that possibly these nitrogen bases were responsible for the observed differences. Table XI demonstrates the validity of this assumption in that as the concentration of adenine, guanine, and uracil was increased a progressive decrease in the titration values was obtained both in the absence of serine and in the presence of limiting serine. Individual and combined effects of adenine, guanine, xanthine, and uracil, each at a concentration of 0.5 mg. per tube as indicated, are demonstrated by Figure 7. From Curve B of Figure 7 it is apparent that adenine is the most inhibitory toward serine synthesis by the organism and xanthine seems to be neither stimulatory nor toxic. Guanine and uracil are also inhibitory to serine synthesis but to a lesser degree than adenine.

Further evidence of the effect of adenine, guanine and uracil on serine synthesis is reflected in the data presented in Table XII. It has been shown that S. faecalis was essentially completely inhibited by a ratio of threonine to serine of 2500 under specific conditions. The titration of 1.32 ml. of 0.1 N sodium hydroxide given under 0.05 γ folic acid column of Table XII represents growth response obtained under such conditions. It is to be noted as these conditions were changed, with respect to the purines and uracil, there was a change in the titration values. Adenine alone has practically the same inhibitory effect as a combination of adenine, guanine and uracil. However, the addition of either guanine or uracil or the omission of these nitrogen bases has a pronounced effect on the titration values. The increase in titration values given under 0.05 γ folic acid column (Table XII) clearly indicated that the ratio of threonine to serine of 2500 was no longer inhibitory when the medium was changed with respect to the purines and uracil.

TABLE XI

INHIBITORY EFFECT OF ADENINE, GUANINE, AND URACIL

Adenine, guanine, and uracil	No serine	20 γ DL-serine
	Titration, 0.1 N NaOH	
mg.[1]	ml.	ml.
0.0	4.04	5.92
0.05	1.60	5.50
0.10	0.75	5.33
0.20	0.43	4.75
0.30	0.15	4.63
0.40	0.06	3.75
0.50	0.0	3.31
0.75	0.0	1.65
1.00	0.0	0.0

[1]The concentrations represent the amount of each constituent present.

Folic acid, 0.05 γ per 10 ml. tube.

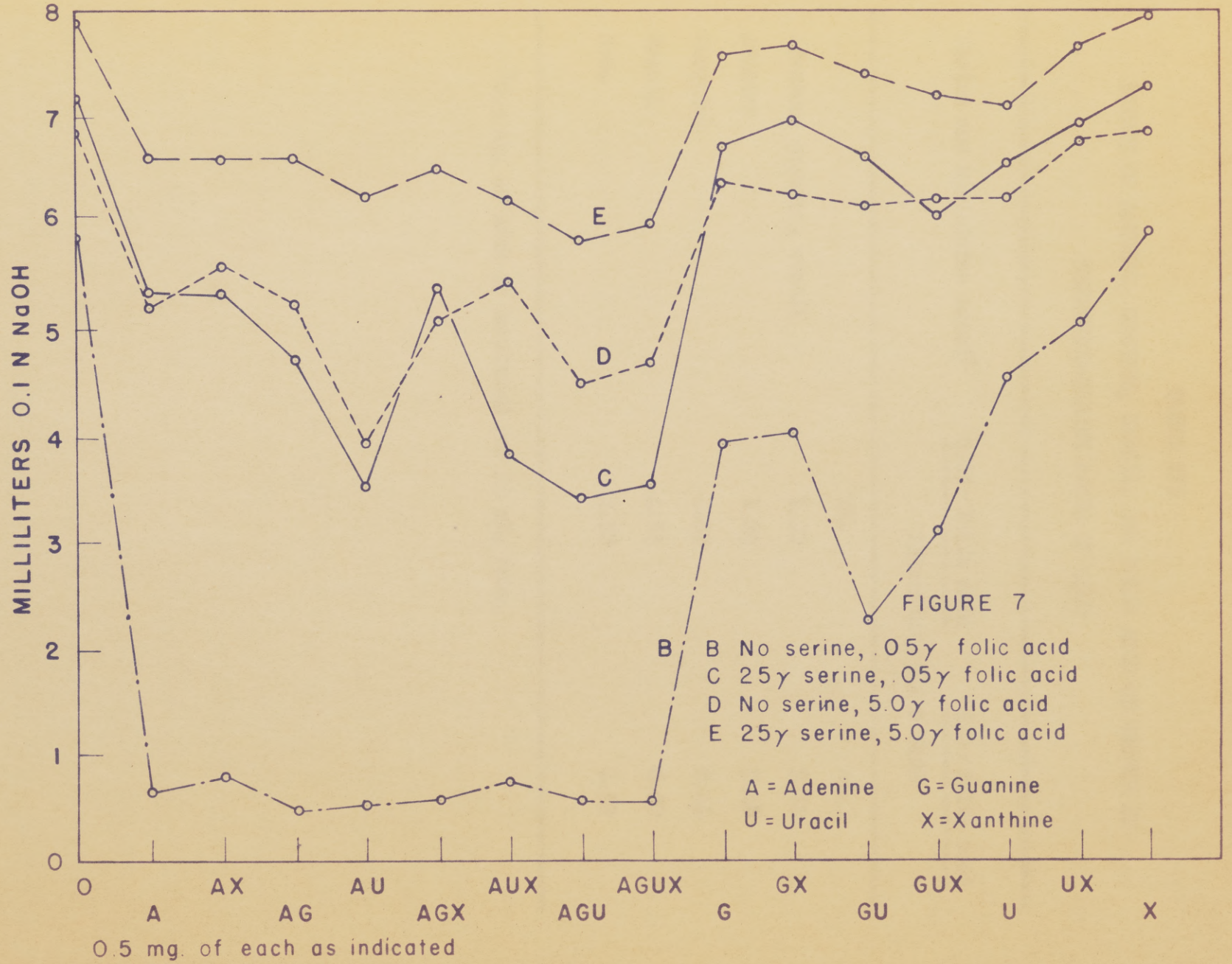

MILLILITERS 0.1 N NaOH
8
7
6
5
4
3
2
1
0
E
D
C
B
O
AX
AU
AUX
AGUX
GX
GUX
UX
A
AG
AGX
AGU
G
GU
U
X
0.5 mg. of each as indicated
B No serine, .05γ folic acid
C 25γ serine, .05γ folic acid
D No serine, 5.0γ folic acid
E 25γ serine, 5.0γ folic acid
A = Adenine
G = Guanine
U = Uracil
X = Xanthine

FIGURE 7

TABLE XII

EFFECT OF ADENINE, GUANINE, URACIL, AND FOLIC ACID ON GROWTH AT THREONINE-SERINE RATIO OF 2500

Purine and Pyrimidine added[2]	0.05γ Folic acid	5.0γ Folic acid
	Titration, 0.1 N NaOH	
	ml.	ml.
Adenine, guanine, uracil	1.32	3.74
Adenine	1.84	5.48
Guranine	4.85	6.44
Uracil	4.94	6.00
None	5.52	6.59

[2]0.5 mg. of each as indicated per 10 ml. tube.

An extension of the study of the purine and uracil toxicity with threonine and valine (Table XIII) indicated that this toxicity was not present under conditions where no threonine or valine was added, and under conditions of limiting threonine or valine. A comparison of the titration values in Table XIII verified that the response of S. faecalis R to threonine and valine was essentially independent of the purines and uracil concentration; whereas, growth of this microorganism was decreased as the concentration of adenine, guanine, and uracil was increased in the absence of serine or in the presence of limiting amounts of serine. In the threonine and valine studies, 0.1 mg. of adenine, guanine, and uracil per tube exerted a slight stimulatory effect on growth rather than an inhibition of growth as with serine studies. From these data, it seems that the inhibitory effect of adenine, guanine, and uracil is specific for serine metabolism in S. faecalis R.

Folic Acid. Early in the study of the toxicity of adenine, guanine, and uracil on serine metabolism by S. faecalis R, it was deemed desirable to correlate this observation with the concentration of folic acid present in the assay tubes. Evidence of a relationship between folic acid and the pyrimidine, thymine, had been published earlier by Stokes (53). Snell and Mitchell (51) had also shown that S. faecalis R required both adenine and thymine in the absence of folic acid. All curves presented on Figure 7 were obtained at the same time; that is, basal medium, inoculum, incubation time, and sodium hydroxide for titration were the same for all tubes which made up the points shown on the curves. Points for Curve B were obtained on a medium containing no serine and 0.05γ folic acid per 10 ml. tube; whereas, the medium for Curve D contained 5.0γ folic acid. A comparison of these two curves showed that the

TABLE XIII

RESPONSE TO SERINE, THREONINE, AND VALINE AS INFLUENCED BY ADENINE, GUANINE, URACIL, AND FOLIC ACID

Amino Acid		Folic acid	Adenine, guanine, and uracil		
			None	0.1 mg.	1.0 mg.
			Titration, 0.1 N NaOH		
	mg.	γ	ml.	ml.	ml.
DL-Serine	0.0	0.05	5.00	2.58	0.78
	0.05	0.05	7.60	7.48	4.96
	0.0	5.0	6.48	5.40	1.88
	0.05	5.0	7.95	8.10	7.20
DL-Threonine	0.0	0.05	1.00	1.25	1.00
	0.05	0.05	5.30	5.65	5.10
	0.0	5.0	1.05	1.25	0.95
	0.05	5.0	4.92	5.80	5.60
DL-Valine	0.0	0.05	1.70	2.00	1.65
	0.05	0.05	4.94	5.46	5.16
	0.0	5.0	1.70	1.85	1.90
	0.05	5.0	4.65	5.40	5.35

higher level of folic acid caused an increase in growth under all combinations of adenine, guanine, uracil, and xanthine. This increase in growth was practically equal to the increase in growth obtained by adding 25γ DL-serine to tubes containing 0.05γ folic acid (Curve C, Figure 7). The addition of 25γ DL-serine to tubes containing 5.0γ folic acid (Curve E, Figure 7) gave greater growth than that obtained with either folic acid or serine alone. A change in the inhibition ratio of threonine to serine of 2500 is indicated by the increase in titration obtained by raising the folic acid from 0.05γ to 5.0γ per 10 ml. tube (Table XII).

Concentrations of folic acid of 0.05γ per tube (31) and 0.1γ per tube (20), which are satisfactory under most assay conditions, do not suffice with limiting serine. Table XIII presents data to show that increased folic acid requirement is restricted to the serine blanks and limiting serine tubes. As indicated by the titrations given in Table XIII, threonine and valine blanks and tubes containing limiting threonine and valine were not stimulated by increasing the folic acid from 0.05 to 5.0γ per tube. However, titrations of serine blanks and limiting serine tubes were increased by such an increase in folic acid. This folic acid effect with serine conditions as given above extended to all concentrations given in Table XIII.

In arriving at a level of folic acid for the preceding work, it was observed that high concentrations of this vitamin were toxic. Table XIV presents data showing the growth response obtained. A study of the folic acid toxicity was extended to a folic acid concentrate[3]. Titrations given in the table for

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[3]A concentrate of "potency" 4000 prepared from spinach. Obtained through the courtesy of Dr. R. J. Williams, University of Texas.

TABLE XIV

TOXICITY TEST FOR FOLIC ACID AND FOLIC ACID CONCENTRATE

Folic acid[4]	Titration 0.1 N NaOH	Folic acid[5]	Titration 0.1 N NaOH	Folic acid[5] concentrate	Titration 0.1 N NaOH
γ	ml.	γ	ml.	γ	ml.
0.00	0.00	0.00	0.40	0	0.72
0.05	0.00	0.05	3.60	2	5.71
0.50	1.50	0.50	6.33	20	7.20
5.00	3.58	5.00	6.80	200	7.40
50.00	3.10	50.00	6.19	800	7.91
500.00	0.78	500.00	2.65	2000	8.40
2500.00	0.49	2500.00	0.66	20000	9.30

[4]0.5 mg. each of adenine, guanine, and uracil present.

[5]0.1 mg. each of adenine, guanine, and uracil present.

folic acid, obtained in the presence of both 0.1 mg. and 0.5 mg. adenine, guanine, and uracil, showed that the toxicity of folic acid started to exert itself between 5.0 and 50 γ. Independent studies not shown gave equal titration values from 2.5 to 10 γ folic acid. The folic acid concentrate did not exhibit the same toxic effect as evidenced by folic acid. The concentrate preparation up to 20 mg. per 10 ml. tube failed to exert a retardation on the growth of S. faecalis R. To the contrary, there was progressive increase in growth as the amount of concentrate was increased.

The reversal of the folic acid toxicity was accomplished by folic acid concentrate and serine as shown in Table XV. The ability of the concentrate to reverse the toxicity of high concentrations of folic acid is probably due to serine contamination in the crude preparations. Support was given to this idea because an acid hydrolysate of the folic acid concentrate retained its ability to reverse the toxicity exerted by folic acid.

Citrate and Fe^{++}. Another apparent difference between Media A, B, and C (Table X) was the presence of sodium citrate as a buffer in Medium A but not in the other two. The superiority of citrate as a buffer had been claimed by several workers (20, 35, 56). In a study of the mineral requirements of S. faecalis R (MacLeod and Snell (35)) and in the leucine assay by this microorganism (Henderson and Snell (20)), citrate buffer did not increase the need for Salts B. Yet, under serine blank conditions the addition of increasing amounts of Salts B (Table XVI) and specifically Fe^{++} (Tables XVI and XVII) overcame the toxicity exerted by increasing amounts of citrate. Under the same conditions, Mg^{++} and Mn^{++} had no effect in reversing the toxicity of sodium citrate.

TABLE XV

REVERSAL OF FOLIC ACID TOXICITY BY SERINE AND FOLIC ACID CONCENTRATE

Folic acid	DL-Serine		Folic acid concentrate	
		Titration, 0.1 N NaOH		Titration, 0.1 N NaOH
γ	γ	ml.		ml.
500	0	2.03	20	2.16
500	25	5.07	100	3.10
500	50	6.47	200	3.66
500	100	8.18	500	4.50
500	200	9.28	1000	5.41
5	0	5.40	20	6.66
5	25	6.75	100	6.71
5	50	7.61	200	6.85
5	100	8.70	500	7.49
5	200	9.60	1000	8.11

0.1 mg. each of adenine, guanine and uracil per tube.

TABLE XVI

EFFECT OF Fe^{++}, Mn^{++}, AND Mg^{++} ON SERINE BLANK

Salts B	Titration, 0.1 N NaOH	Fe^{++}	Mn^{++}	Mg^{++}	Titration, 0.1 N NaOH
ml.	ml.	γ	γ	γ	ml.
0.0	1.35	20	25	200	1.35
0.05	1.35	50	25	200	1.60
0.0625	1.55	100	25	200	2.00
0.125	2.30	500	25	200	4.45
0.3125	3.33	20	50	200	1.35
0.625	4.48	20	100	200	1.30
1.25	4.42	20	500	200	1.40
		20	25	500	1.32
		20	25	800	1.88
		20	25	1000	1.30

2 per cent citrate, 5.0 γ of folic acid, and 0.5 mg. each of adenine, guanine, and uracil present per tube.

Fe^{++} as $FeSO_4.7H_2O$; Mn^{++} as $MnSO_4.H_2O$; Mg^{++} as $MgSO_4.7H_2O$.

TABLE XVII

EFFECT OF CITRATE AND Fe^{++} ON SERINE BLANK

Citrate	20 γ Fe^{++}	500 γ Fe^{++}
	Titration, 0.1 N NaOH	
mg.	ml.	ml.
0	3.68	3.40
25	3.50	3.80
50	3.20	4.25
100	2.62	4.64
200	2.62	5.10

60 mg. sodium acetate, 5.0 γ folic acid, and
0.5 mg. each of adenine, guanine, and uracil
per 10 ml. tube.

Discussion. The diversity of the factors which have been shown to alter the serine requirement of S. faecalis R makes it difficult to formulate a scheme which includes all of the findings. However, consideration of these data with that already published permits the formulation of a pattern which includes all of the factors presented. Considerable work has been reported showing a relationship between thymine and folic acid. Snell and Mitchell (51), and Stokstad (55) found that thymine and the purines were capable of promoting the growth of S. faecalis R in the absence of added folic acid. Stokes (53) suggested that folic acid functions as a coenzyme for the enzyme system responsible for the synthesis of thymine, or a thymine-like compound, which was in turn used by the bacteria to form nucleic acids. Hall (18), in reporting the synergistic effect of thymine and folic acid for S. faecalis R, postulated that thymine may actually be a precursor of folic acid, or thymine is participating in some alternative metabolic path. Rogers and Shive (47), interpreted the data of Hall (18) as a sparing action of thymine on folic acid. When thymine was added to the medium, the folic acid requirement of the organism was lowered because in the presence of thymine less or no folic acid was required for the synthesis of thymine or a thymine-like compound. The analogue-metabolite inhibition studies by Rogers and Shive (47) with L. casei indicated that the function of folic acid was that of a coenzyme or as a source in the production of a coenzyme which was necessary for the synthesis of the purines and thymine or their equivalents.

Even though the present findings are not entirely in keeping with the conclusions given above, the fact remains that adenine exerts a toxicity toward

serine synthesis by S. faecalis R and that both serine and folic acid reverse this toxicity. The role of adenine as an inhibitor is best explained by assuming that this compound inhibits the functioning of some enzyme responsible for the synthesis of serine. This inhibition possibly being exerted because of the similarity in structure between adenine and a specific part of the enzyme, probably purine-like or perhaps similar in structure to the pyrimidines. In addition to guanine, uracil, and xanthine, both thymine and hypoxanthine show no reversal of adenine toxicity. This indicates that these compounds are probably not part of the enzyme responsible for the synthesis of serine.

The reversal of adenine, guanine, and uracil toxicity by folic acid and serine suggests that folic acid is part of an enzyme system responsible for the synthesis of serine by S. faecalis R. As the organism is used in assay for folic acid in the presence of serine, it would not be a matter of serine being converted to folic acid which would then be responsible for the reversal of the adenine toxicity. The inhibitory effect of high concentrations of folic acid and the reversal of this toxicity by both serine and folic acid concentrate from spinach is, however, suggestive of a special form of folic acid being necessary for serine synthesis. To explain the toxicity of folic acid, it is to be assumed that the organism has the ability to convert pteroylglutamic acid into the desired form as part of the enzyme for serine synthesis. However, as the concentration of pteroylglutamic acid is increased it becomes competitive with the specific form because of the excess of pteroylglutamic acid present. That is, with sufficient pteroylglutamic acid, 5.0 γ per 10 ml. tube, the organism has the ability to convert a sufficient amount for serine

synthesis. However, in the presence of excess pteroylglutamic acid this conversion rate may be the same but the presence of excess pteroylglutamic acid competes with the special form of folic acid formed and serine synthesis is blocked. The reversal of pteroylglutamic acid toxicity by folic acid concentrate is believed to be due to the presence of serine in the concentrate because an acid hydrolysate of this concentrate retains its ability to reverse the toxicity of pteroylglutamic acid. Serine assays with L. delbrueckii, in the absence of threonine, indicate the presence of serine in an acid hydrolysate of the concentrate.

The inhibitory effect of citrate on the growth of S. faecalis R seems to be due to the complex formation of citrate with the bivalent cation, Fe^{++}. Table XVII indicates the inhibitory effect of increasing amounts of citrate in the presence of 20 γ Fe^{++}, the amount supplied by 0.05 ml. Salts B. Data presented in this table also show the reversal of citrate toxicity in the presence of 500 γ Fe^{++} per 10 ml. tube. Henderson and Snell (20) had previously shown that S. faecalis R did not require extra Salts B for leucine assays on a citrate buffered medium. However, Table XVI presents data which show an enhancement of growth of this organism, under serine blank conditions in citrate buffered medium, as the volume of Sats B per tube is increased. The specifity of Fe^{++}, when compared to Mn^{++} and Mg^{++}, in increasing growth in serine blank tubes is also revealed in this table.

The data presented on the influence of Fe^{++} on serine blanks with S. faecalis R suggest that this cation is essential to the synthesis of serine. That is, Fe^{++} seems to be an activator for the enzyme responsible for the synthesis of serine or it may be an integral part of the enzyme system proper.

The role of citrate as a inhibitor appears to be that of removing Fe^{++} from the medium by complex formation and thereby preventing the synthesis of serine.

All of the findings presented have been interpreted on the postulation of an enzyme system present in S. faecalis R which is responsible for the synthesis of serine. The toxicity of adenine, and the reversal of this toxicity by serine, suggests that one part of the enzyme has a structure similar to the purines. The data presented on folic acid indicate that a compound related to folic acid in structure is part of the enzyme responsible for the synthesis of serine. The toxicity of citrate under limiting serine conditions and the reversal of this toxicity by Fe^{++} suggests that possibly Fe^{++} is an activator of the serine forming enzyme system. Because of the many factors which influence the serine requirement of S. faecalis R, it is doubtful if this microorganism should be used in the assay for serine.

CHAPTER VI

STUDIES ON THE CALCIUM REQUIREMENT OF

LACTOBACILLUS DELBRUECKII-5

STUDIES ON THE CALCIUM REQUIREMENT OF LACTOBACILLUS DELBRUECKII-5

Sodium citrate and sodium succinate, because of their greater buffer capacity when compared to sodium acetate, have come to be used in assay media for the lactic acid bacteria. The growth of some of the members of this group of bacteria is adversely affected when the pH of the medium falls below 5.2. Such conditions, because of the lactic acid produced by the bacteria, are readily attained in assay tubes which are not properly buffered. The increased buffer capacity of citrate and succinate are sufficient to maintain the pH above 5.2 over a greater growth range, hence the assay range is increased.

The use of citrate in the medium, however, causes part of the Mn^{++} and Mg^{++} of the medium to become unavailable to the microorganism due to the complex formation between citrate and the two cations, Mn^{++} and Mg^{++} (35). To overcome this effect more magnesium and manganese salts must be added to the basal medium. As demonstrated in the preceding chapter, the Fe^{++} requirement of S. faecalis R was also increased in the presence of citrate buffer. Lyman and coworkers have used succinate as a buffer for S. faecalis R in the determination of methionine (31), tryptophan (27), and histidine (30) and have reported no difficulty with the use of succinate as a buffer. Yet, in the present investigation when either citrate or succinate was used as a buffer for L. delbrueckii in serine assays the growth of the organism was inhibited.

Toxicity of Citrate. Evidence of the difference in the response of L. delbrueckii on two different media, both free of threonine, is given in Table XVIII. The medium of Henderson and Snell (20) caused an appreciable

TABLE XVIII

RESPONSE OF L. DELBRUECKII TO SERINE ON MEDIA OF HENDERSON AND SNELL (20), AND STOKES, GUNNESS, DWYER AND CASWELL (54)

DL-Serine	Medium of Henderson and Snell	Medium of Stokes et al
	Titration, 0.1 N NaOH	
γ	ml.	ml.
0	0	0.50
5	0	1.20
10	0.20	1.85
20	0.34	3.06
40	0.20	4.98
100	1.10	8.20

lag in the response to serine even though threonine was omitted, while with the medium of Stokes et al (54) the lag section was relatively small. To determine the cause of the lag, the media given above were each made up in three parts, amino acids, vitamin supplements, and buffers, and combined as given in Table XIX. From the data presented in this table, it was apparent that the salts and buffer mixture H (Henderson and Snell) was responsible for the inhibition of growth. By further division of the buffer and salts mixtures H and S (Stokes et al) as given in Table XX, it was possible to establish the inhibitory effect of citrate.

A comparison of citrate and succinate at different levels, in the presence of different levels of salts B (Table XXI) showed citrate to be more toxic than succinate under all conditions given. From these data, it was noted that by increasing the concentration of salts B, there was a tendency toward reversal of the citrate and succinate toxicity. However, no concentration of the salts B used brought the titration of citrate or succinate tubes up to the titration of the tube containing only sodium acetate as a buffer. Individual and combined effects of Mg^{++} and Mn^{++} (Table XXII), likewise failed to remove the inhibitory effect of either sodium citrate or sodium succinate. As with increasing amounts of salts B, only a trend toward reversal of the inhibition was noted as the concentration of Mn^{++} and or Mg^{++} was increased.

Effect of Ca^{++} on Buffer Toxicities with Limiting Serine Conditions. The limited ability of increasing amounts of salts B and of increasing amounts of Mn^{++} and Mg^{++} to reverse the inhibitory effect of citrate and succinate suggested the presence of an impurity in the salts employed that was in reality

TABLE XIX

GROWTH RESPONSES OF L. DELBRUECKII WITH DIFFERENT AMINO ACID, VITAMIN AND BUFFER SUPPLEMENTS

Amino Acids	Vitamins	Buffer and Salts	Titration ml.
H	H	H	0
H	S	H	0
H	H	S	3.24
H	S	S	2.72
S	S	S	3.86
S	H	S	5.25
S	S	H	0
S	H	H	0

"H" signifies amino acids, vitamins, and buffer and salts, respectively, according to the formula of Henderson and Snell (20).

"S" signifies amino acids, vitamins, and buffer and salts, respectively, according to the formula of Stokes et al (54).

Buffer and salts "H": K_2HPO_4, 50 mg.; sodium acetate, 10 mg.; sodium citrate, 200 mg.; ammonium chloride, 30 mg.; salts C, 0.2 ml.

Buffer and salts "S": sodium acetate, 60 mg.; salts A, 0.05 ml.; salts B, 0.05 ml.

Conditions common to all tubes: 50 γ DL-serine; 0.1 mg. each of adenine, guanine, uracil, and xanthine; 200 mg. glucose; no threonine.

TABLE XX

COMPARISON OF EFFECT OF CONSTITUENTS OF BUFFER AND SALT MIXTURES H AND S ON GROWTH OF L. DELBRUECKII

Salts A-0.05 ml. + Salts B-0.05 ml.	Salts C-0.2 ml. + K_2HPO_4 -50 mg.	Sodium Citrate	Ammonium Chloride	Sodium Acetate	
				60 mg.	10 mg.
				Titration	
		mg.	mg.	ml.	ml.
S		0	0	8.25	5.75
	H	0	0	7.43	6.30
S		200	0	0.34	0.28
	H	200	0	0.22	0.25
S		0	30	8.05	5.45
	H	0	30	6.36	6.58
S		200	30	0.40	0.40
	H	200	30	0.60	1.25

Medium of Stokes et al (54), threonine omitted, with vitamin supplement of Henderson and Snell (20). Serine-DL 50γ per tube.

TABLE XXI

INFLUENCE OF CONCENTRATION OF SALTS B ON GROWTH OF L. DELBRUECKII

Salts B	Sodium Citrate or Sodium Succinate	Sodium Citrate	Sodium Succinate
		Titration, 0.1 N NaOH	
ml.	mg.	ml.	ml.
0.05	0	6.07	5.94
0.05	25	2.20	5.60
0.05	50	0.85	5.28
0.05	100	0	4.76
0.05	200	0	2.63
0.5	0	6.45	6.45
0.5	25	2.92	6.10
0.5	50	1.30	5.30
0.5	100	0	5.20
0.5	200	0	3.60

Medium of Stokes et al (54) with threonine omitted.

DL-serine 50 γ per tube.

TABLE XXII

Mg^{++} AND Mn^{++} INFLUENCE ON CITRATE AND SUCCINATE TOXICITY

Sodium Citrate or Sodium Succinate	Mg^{++}	Mn^{++}	Sodium Citrate Titration, 0.1 N NaOH	Sodium Succinate Titration, 0.1 N NaOH
mg.	mg.	mg.	ml.	ml.
0	0.2	0.025	6.21	6.21
200	0.2	0.025	0	2.95
200	0.7	0.025	0	3.20
200	1.2	0.025	0	3.42
200	2.2	0.025	0.5	2.84
200	5.2	0.025	1.0	2.70
200	10.2	0.025	1.84	2.56
200	0.2	0.525	0	4.15
200	0.2	1.025	0	4.36
200	0.2	2.025	0	4.70
200	0.2	5.025	0	2.98
200	0.2	10.025	0.2	4.00
200	1.2	1.025	1.82	1.00
200	5.2	1.025	3.00	5.00
200	10.2	1.025	3.42	4.78
200	5.2	1.025	3.12	4.84
200	5.2	5.025	4.00	5.50
200	5.2	10.025	4.90	5.78

Medium of Stokes et al (54) with threonine omitted.

50 γ DL-serine per tube.

responsible for the effects obtained. Although analytical grades of salts were used, calcium analyses given on the labels were of sufficient magnitude to warrant an investigation of the influence of Ca^{++} on the growth of L. delbrueckii. Another indication of the possible need for calcium is given in Table XIX. Vitamin supplement H contained 10 γ calcium pantothenate; whereas, vitamin supplement S contained only 2 γ of this vitamin. It is to be noted that in the cases where vitamin supplement H was used, other constituents being the same, the resulting growth was greater. Table XXIII shows the stimulatory effect of Ca^{++} in a medium containing succinate as a buffer. The addition of 200 mg. of sodium succinate to an acetate buffered medium lowered the growth titration from 6.15 ml. to 4.00 ml. of 0.1 N sodium hydroxide. The addition of 0.05 mg. Ca^{++} to a succinate buffered tube raised the titration above that of the acetate buffered tube, a titration of 6.45 ml. with Ca^{++} added as compared to a titration of 6.15 ml. with no Ca^{++} added. The titration of a succinate buffered tube was further raised when 0.1 mg. Ca^{++} was added, but was lowered by the addition of 0.5 mg. Ca^{++}. The data presented in Table XXIV shows that by the addition of as little as 10 γ Ca^{++} the titrations for acetate and succinate buffered tubes are increased; whereas, the citrate buffered tubes produced no growth. The absence of growth in the citrate buffered tubes is possibly explainable on the fact that Mn^{++} and Mg^{++} were limiting because only 0.05 ml. salts B were added per tube. Evidence of the greater complex formation in the presence of citrate buffer was noted in the tubes containing 0.2 and 0.5 mg. Ca^{++}. With the succinate and acetate buffer, a precipitate formed when the tubes were autoclaved but with citrate

TABLE XXIII

INFLUENCE OF Ca^{++} ON RESPONSE OF L. DELBRUECKII TO SERINE ON SUCCINATE BUFFERED MEDIUM

DL-Serine	Sodium Succinate	Ca^{++}	Titration, 0.1 N NaOH
γ	mg.	mg.	ml.
50	0	0	6.15
50	200	0	4.00
50	200	0.05	6.54
50	200	0.10	7.50
50	200	0.50	6.75
50	0	0.05	6.35
50	0	0.10	6.25
50	0	0.50	6.18

Medium of Stokes et al (54) with 60 mg. sodium acetate common to all tubes.

Ca^{++} as $CaCl_2.2H_2O$

TABLE XXIV

EFFECT OF Ca^{++} ON GROWTH OF L. DELBRUECKII IN PRESENCE OF DIFFERENT BUFFERS

DL-Serine	Ca^{++}	Sodium Acetate		Sodium Succinate	Sodium Citrate
		60 mg.	200 mg.	200 mg.	200 mg.
		Titration, 0.1 N NaOH			
γ	mg.	ml.	ml.	ml.	ml.
50	0.0	7.95	5.25	5.85	0
50	0.0	7.98	5.42	6.82	0
50	0.01	8.71	6.68	8.00	0
50	0.025	8.60	8.30	8.78	0
50	0.05	8.70	9.30	9.02	0
50	0.10	8.55	9.65	9.60	0
50	0.20	8.40	9.50	9.70	0
50	0.50	8.50	9.12	10.30	0

Medium of Stokes et al (54) with vitamin supplement of Henderson and Snell (20) with the exception that 2 γ calcium pantothenate was used instead of 10 γ.

Sodium acetate 10 mg. and glucose 200 mg. per tube.

no such precipitate formed. A comparison of salts B (0.05 ml. per tube) and salts C (0.2 ml. per tube) in citrate buffered tubes, Table XXV, showed an increased growth response where salts C was used. However, the addition of Ca^{++} further increased the growth even in the presence of salts C.

Because of the inhibitory effects of acetate, succinate, and citrate, all of which have been shown to be important in the intermediary carbohydrate metabolism, it was thought that the use of these compounds at high concentrations was causing the inhibitory effect by shifting certain equilibria in the carbohydrate degradation. To test such a hypothesis, succinate, acetate, and citrate were compared with saccharate as a buffer. The latter compound has not been postulated as an intermediate in carbohydrate metabolism and therefore should not have an influence if the above hypothesis was valid. Table XXVI shows that sodium potassium saccharate exhibits an inhibitory effect on the growth of L. delbrueckii similar to that of the other buffers. This indicates that the inhibitory effect of these buffers was not directly influencing the carbohydrate metabolism. The stimulatory effect of Ca^{++} was again apparent.

Effect of Ca^{++} with Different Limiting Amino Acid Conditions. The results presented so far have been obtained under limiting serine conditions, 50 to 200 γ DL-serine per 10 ml. tube. These results have indicated a need for extra calcium in preference to extra Mg^{++} and Mn^{++} in the presence of the organic acid buffers studied. Table XXVII shows the influence of Ca^{++} on leucine utilisation in comparison to serine utilization. In general, the data indicates a requirement for calcium. However, the Ca^{++} required for limiting leucine conditions is smaller both in the presence of succinate and citrate. Also, there

TABLE XXV

EFFECT OF Ca^{++} ON THE RESPONSE OF *L. DELBRUECKII* TO SERINE ON CITRATE BUFFERED TUBES CONTAINING SALTS B AND SALTS C

			0.05 ml. Salts B		0.2 ml. Salts C	
Ca^{++}	DL-Serine		Titration, 0.1 N NaOH			
mg.	γ	γ	ml.	ml.	ml.	ml.
0	50	100	0.0	0.0	1.30	1.25
0.1	50	100	0.0	0.0	1.70	4.91
0.2	50	100	0.18	0.15	2.65	6.38
0.5	50	100	0.15	0.30	5.62	8.78
1.0	50	100	0.35	3.62	8.05	11.05

Medium same as for Table XXIV except 0.1 mg. xanthine added per tube and medium contained 2 per cent glucose.

No threonine in the medium.

TABLE XXVI

COMPARISON OF DIFFERENT BUFFERS FOR L. DELBRUECKII

DL-Serine	Ca^{++}	Buffer	Sodium Acetate	Sodium Citrate	Sodium Succinate	Sodium Potassium Saccharate
			Titration, 0.1 N NaOH			
γ	mg.	mg.	ml.	ml.	ml.	ml.
50	0	50	7.42	5.95	6.88	6.00
50	0	100	7.98	5.70	7.30	5.40
50	0	200	6.50	1.88	7.68	5.10
2000	0	100	17.90	17.70	16.48	13.95
50	0.2	50	9.42	8.52	8.40	7.20
50	0.2	100	11.40	7.80	10.00	7.36
50	0.2	200	11.10	4.00	11.40	7.52
2000	0.2	100	17.34	17.75	16.60	13.50

Medium same as for Table XXV with 0.2 ml. salts C per tube.

TABLE XXVII

RESPONSE OF L. DELBRUECKII TO SERINE AND LEUCINE AS INFLUENCED BY Ca^{++}

DL-Leucine	DL-Serine	Titration-ml. 0.1 N NaOH		
γ	γ	No Ca^{++}	0.2 mg. Ca^{++}	0.5 mg. Ca^{++}
200 mg. sodium citrate per tube				
2000	50	0	0	0
2000	100	0	0	0
2000	200	0.38	0.37	0.70
2000	400	0.72	0.68	2.60
2000	2000	9.83	16.50	17.08
50	2000	3.56	4.30	4.53
100	2000	5.52	6.30	6.80
200	2000	6.50	9.50	10.03
200 mg. sodium succinate per tube				
2000	50	0	2.30	4.50
2000	100	0.70	--	12.11
2000	200	1.80	13.75	16.21
2000	400	7.16	19.10	19.62
2000	2000	18.93	19.31	19.71
50	2000	5.37	8.45	8.08
100	2000	9.96	10.38	11.00
200	2000	13.60	11.41	11.62

Medium of Henderson and Snell (20).

is an apparent difference between the succinate and citrate buffered tubes containing 2 mg. each of serine and leucine. With citrate, the addition of 0.2 mg. Ca^{++} increased the titration from 9.83 to 16.50; whereas, with succinate the increase in titration was only from 18.93 to 19.31 ml. 0.1 N sodium hydroxide. The titrations obtained on citrate and succinate buffered tubes suggested that the Ca^{++} requirement was partially a function of the media. With citrate as buffer, there was an appreciable lag in the growth response of the microorganism to serine. This lag was much smaller with succinate as buffer. The inhibition of serine utilization by citrate seemed to be extensive enough to make 2000 γ DL-serine per tube a limiting factor for growth. With succinate, the inhibition was less and 2000 γ DL-serine was not limiting and top growth was obtained in the absence of added Ca^{++}.

The results of a study of the effect of Ca^{++} on the utilization of different limiting amino acids are given in Table XXVIII. In general, titrations obtained were greater where Ca^{++} was added. Also given are visual growth observations at 24 and 40 hours. These visual observations are given to demonstrate the fact that tubes containing Ca^{++} gave a greater early growth than tubes containing no added Ca^{++}. This effect of Ca^{++} had been observed in many of the previous experiments.

Because of the extreme toxicity of a citrate buffered medium to the utilization of serine by L. delbrueckii, an experiment was designed to determine the influence of Ca^{++} on the utilization of arginine, leucine, and valine in the presence of varying amounts of serine. Results of the investigation are presented in Table XXIX. In general, as the concentration of serine was increased there was a decreasing difference in the titrations between tubes

TABLE XXVIII

GROWTH OF L. DELBRUECKII UNDER DIFFERENT LIMITING AMINO ACIDS AS INFLUENCED BY Ca^{++}

Limiting Amino Acid		Growth Observations[1]				Titration, 0.1 N NaOH	
		24 hours		40 hours		72 hours	
		No Ca^{++} Added	0.2 mg. Ca^{++}	No Ca^{++} Added	0.2 mg. Ca^{++}	No Ca^{++} Added	0.2 mg. Ca^{++}
		200 mg. Sodium Citrate per tube					
	γ					ml.	ml.
L-Arginine.HCl	50	-	-	+	+	4.63	4.70
L-Histidine.HCl.H_2O	50	-	-	2+	2+	10.36	15.81
DL-Isoleucine	100	-	-	+	2+	9.48	11.23
DL-Leucine	100	-	-	+	2+	7.23	8.70
DL-Phenylalanine	100	-	-	+	2+	6.15	7.74
DL-Serine	1000	-	-	±	+	3.90	6.36
DL-Valine	100	-	-	+	2+	6.84	7.15
None		+	4+	2+	4+	10.24	16.22

TABLE XVIII (continued)

GROWTH OF L. DELBRUECKII UNDER DIFFERENT LIMITING AMINO ACIDS

AS INFLUENCED BY Ca^{++}

Limiting Amino Acid		Growth Observations[1] 24 hours		40 hours		Titration, 0.1 N NaOH 72 hours	
		No Ca^{++} Added	0.2 mg. Ca^{++}	No Ca^{++} Added	0.2 mg. Ca^{++}	No Ca^{++} Added	0.2 mg. Ca^{++}
		200 mg. Sodium Succinate per tube					
	γ					ml.	ml.
L-Arginine.HCl	50	3+	3+	3+	3+	6.44	6.28
L-Histidine.HCl.H_2O	50	-	+	4+	4+	16.80	19.45
DL-Isoleucine	50	-	+	4+	4+	14.46	14.95
DL-Leucine	100	+	3+	2+	3+	8.51	11.00
DL-Phenylalanine	100	3+	3+	3+	3+	9.40	9.96
DL-Serine	1000	+	4+	2+	4+	14.53	18.64
DL-Valine	100	-	+	3+	4+	8.89	9.39
None		-	+	3+	3+	20.36	18.92
None[2]		+	4+	4+	4+	15.07	18.48

[1]No growth, minus; heavy growth, 4-plus.

[2]60 mg. sodium acetate as buffer.

Medium of Henderson and Snell (20).

TABLE XXIX

EFFECT OF SERINE CONCENTRATION ON THE Ca^{++} REQUIREMENT OF L. DELBRUECKII

DL-Serine	L-Arginine HCl	DL-Leucine	DL-Valine	Ca^{++}	Titration, 0.1 N NaOH	
					No Threonine	2 mg. DL-Threonine
mg.	mg.	mg.	mg.	mg.	ml.	ml.
0.1	2	2	2	0	2.60	1.10
0.1	2	2	2	0.2	8.18	1.95
0.5	2	2	2	0	15.88	1.10
0.5	2	2	2	0.2	18.18	3.80
1	2	2	2	0	15.80	2.22
1	2	2	2	0.2	18.59	5.72
2	2	2	2	0	16.73	8.79
2	2	2	2	0.2	19.04	11.50
4	2	2	2	0	17.59	14.61
4	2	2	2	0.2	19.53	18.00
8	2	2	2	0	18.25	15.90
8	2	2	2	0.2	19.03	18.38
2	0.05	2	2	0	5.95	3.84
2	0.05	2	2	0.2	7.40	6.00
4	0.05	2	2	0	6.07	6.10
4	0.05	2	2	0.2	7.65	6.92
8	0.05	2	2	0	6.08	6.32
8	0.05	2	2	0.2	7.85	6.74
2	2	0.1	2	0	10.00	6.10
2	2	0.1	2	0.2	11.87	8.93
4	2	0.1	2	0	10.74	9.90
4	2	0.1	2	0.2	12.55	11.30
8	2	0.1	2	0	11.50	10.20
8	2	0.1	2	0.2	11.85	11.46
2	2	2	0.1	0	9.20	6.08
2	2	2	0.1	0.2	11.50	7.90
4	2	2	0.1	0	10.90	6.88
4	2	2	0.1	0.2	10.73	8.42
8	2	2	0.1	0	10.00	7.34
8	2	2	0.1	0.2	10.48	8.50

Medium of Henderson and Snell (20) except 2γ calcium pantothenate instead of 10γ; amino acids of Stokes et al (54).

containing added Ca^{++} and those containing no added Ca^{++}. Also, greater differences were noted at lower levels of serine between the Ca^{++} and non-Ca^{++} tubes containing threonine when compared to tubes containing no threonine. This phenomenon was attributed to the inhibitory effect of threonine on the utilization of serine. These data and other observations recorded in the preceding tables suggested that L. delbrueckii required more Ca^{++} under a number of conditions in which growth was inhibited. An explanation of the role of buffers and inhibitions in determining the Ca^{++} requirement of L. delbrueckii will be developed in the following discussion.

Discussion of the Ca^{++} Requirement of L. delbrueckii. From the data presented, it is apparent that the Ca^{++} requirement of L. delbrueckii varies with the buffer present in the basal media and the concentration of a particular buffer. For example, L. delbrueckii, in response to 50 γ DL-serine on a medium containing 60 mg. sodium acetate and no added Ca^{++}, produces a growth equivalent to approximately 6 ml. 0.1 N sodium hydroxide. This growth represents approximately two-thirds the maximum growth attainable under optimum medium conditions in the presence of 50 γ DL-serine. If such a growth is used as a basis of comparison for media containing 200 mg. of different buffers, it is indicated that the amount of Ca^{++} needed in the media, above that supplied as impurities, is approximately 0.05 mg. for succinate buffered tubes (Table XXIII), and 0.2 to 0.5 mg. for citrate buffered tubes (Table XXIV). An example of the influence of buffer concentration on the Ca^{++} requirement is shown in Table XXIV. In the presence of 60 mg. sodium acetate per tube, maximum growth is obtained with the addition of only 0.01 mg. Ca^{++}; whereas, with 200 mg. sodium acetate 0.05 to 0.1 mg. Ca^{++} is necessary to produce maximum growth.

The quantitative and the qualitative effects of the buffers on Ca^{++} requirement of L. delbrueckii can be explained by considering the complex forming ability of the buffers employed. Joseph (26) has determined the dissociation constants of organic Ca^{++} complexes and found the Ca^{++} binding power decreased in the order of citrate, succinate, and acetate. The respective values obtained for the dissociation constants for the calcium salts of these three buffers were 3.17, 1.16, and 0.53. These values indicate that citrate has a much greater complex forming ability than either succinate or acetate. Data presented in the paragraph above show that the Ca^{++} requirement decreases in the same order. That is, the Ca^{++} requirement is greatest with citrate, intermediate with succinate, and smallest with acetate. The role of the buffers in determining the Ca^{++} requirement of L. delbrueckii thus seems to be based on the relative abilities of buffers to reduce the Ca^{++} concentration by converting the Ca^{++} into an undissociated form or possibly into an anion complex which cannot be utilized by the organism. By increasing the concentration of the buffer more of the Ca^{++} is complexed and the Ca^{++} requirement is increased.

To explain the need for more calcium, above that furnished as impurities, it is necessary to assume a partial or preferential removal of Ca^{++} by the organic buffers of the media. Although MacLeod and Snell (35) were unable to demonstrate a Ca^{++} requirement for a group of lactic acid bacteria, not including L. delbrueckii-5, they found that Ca^{++} could replace Mn^{++} to a certain extent. This sparing action of Ca^{++} on the Mn^{++} they attributed to the complex formation between Ca^{++} and the citrate buffer of the medium. This

evidence indicates that Ca^{++} is preferentially complexed in comparison to Mn^{++} or that the complex formation is governed by some law of distribution of the Ca^{++} and Mn^{++} in complex formation. The findings of MacLeod and Snell would eliminate the possibility of the preferential binding of Mn^{++} to Ca^{++}.

Although the alkaline earth elements, calcium and magnesium, do not form chelate compounds, because of their failure to share electrons to form covalencies, it has been postulated that there are some other forces which retard the ionization of calcium and magnesium salts of carboxylic acids. According to Pauling (42), the alkaline earth metals form essentially ionic bonds with the more non-metallic elements. Consequently, mainly electrostatic forces can be considered as acting to prevent or retard the ionization of the alkali earth cations. Cannan and Kibrick (7) determined the association constants for calcium and magnesium salts of a number of carboxylic acids and found that Ca^{++} and Mg^{++} are associated to the same extent in many instances. The association constants as determined by these workers for the calcium and magnesium salts of citric, succinic, and acetic acid were 3.22, 1.20 and 0.51 to 0.53, respectively. These values indicate that both Ca^{++} and Mg^{++} would be bound to the same extent by different buffers if they were present in the same concentration. However, from the experimental data demonstrating a need for extra Ca^{++}, above that furnished as impurities, it seems that the small amount of Ca^{++} furnished as impurities is effectively bound by the buffers under consideration. This binding or complexing of the Ca^{++} causes this cation to be unavailable to the microorganism, and more must be added to the medium.

Data presented in Table XXVIII disclose that citrate is more toxic than succinate. From the visual observations recorded in this table, it is apparent

that citrate exerts an appreciable inhibition on the initial growth of the organism. With citrate buffer, no visible growth was noted at 24 hours for any of the limiting amino acid conditions given, but with succinate there was appreciable growth at this time. Also indicated by the data in Table XXVIII is the growth initiation effect of Ca^{++}. With citrate buffer, the tube which was complete with respect to the amino acids showed a plus growth at 24 hours where Ca^{++} was absent and a 4-plus growth in the presence of 0.2 mg. Ca^{++}.

In addition to the lag in the growth response of L. delbrueckii to different limiting amino acids caused by the buffers, threonine also caused an inhibition of growth. A comparison of the growth obtained in tubes containing no threonine with those containing 2 mg. DL-threonine, Table XXIX, shows the inhibitory effect of threonine. However, this inhibitory effect of the threonine seems most pronounced at low levels of serine. This suggests that threonine is blocking the utilization of serine to the extent that 2 mg. DL-serine becomes a limiting factor for the growth of the organism. It is to be noted from Table XXIX that as the serine concentration is increased there is a decrease in the difference in titration between Ca^{++} and non-Ca^{++} containing tubes and between tubes containing threonine and those containing no threonine. The limiting nature of 2 mg. DL-serine per 10 ml. tube is also evidenced in the tests involving arginine, leucine, and valine.

The data presented on different limiting amino acid growth responses, even in the presence of 4 to 8 mg. DL-serine, demonstrate a Ca^{++} requirement for L. delbrueckii. However, the Ca^{++} requirement becomes smaller as the lag in the initiation of growth is removed. This influence of inhibitions on the growth of L. delbrueckii is again explainable on the basis of a complex formation

between Ca^{++} and the organic buffers and the relative stability of these complexes with reference to the pH of the medium. It has been demonstrated by Bobtelsky and Jordan (6) that the stability of some citrate complexes with bi-valent cations decreases with decreasing pH and that the carboxyl group of the organic acid must be in the ionized form before complexing would occur with the bi-valent cations. Both of these facts lend themselves to an explanation of the variable Ca^{++} requirement of L. delbrueckii. As the microorganism grows, it produces lactic acid. This production of lactic acid results in the lowering of the pH of the medium which liberates Ca^{++} from the complexes. With this pH shift the carboxylic group is shifted toward the undissociated form and complex formation ability is further reduced.

Table XXX presents the results of a study on the effect of the pH of the medium on the Ca^{++} requirement of L. delbrueckii. A medium was prepared, divided into four equal parts and adjusted to pH of 5.5, 6.0, 6.5, and 7.0. These media were then added to tubes containing serine, or serine and calcium as given by Table XXX. An uninoculated tube from each medium was also prepared and autoclaved with the experimental tubes. As shown in the table, there were pH shifts of 5.5 to 5.8, 6.0 to 6.3, 6.5 to 6.7, and 7.0 to 7.05. All titrations for a particular media were taken to the pH of the corresponding uninoculated, autoclaved tube. Thus the titration values obtained represented actual growths.

The data presented in Table XXX adequately demonstrates the effect of pH on the Ca^{++} requirement of L. delbrueckii. Growth titration values obtained after 72 hours with tubes containing 200 γ DL-serine at pH 5.3 and 6.3 indicate that the Ca^{++} supplied as impurities is sufficient to meet the requirements of

TABLE XXX

EFFECT OF pH OF MEDIUM ON Ca^{++} REQUIREMENT OF L. DELBRUECKII

DL-Serine	Ca^{++}	Titration Time	Titration, 0.1 N NaOH, Titrated to pH			
			5.8[3]	6.3[3]	6.7[3]	7.05[3]
γ	mg.	hours	ml.	ml.	ml.	ml.
100	0	46	0.30	0.48	0.10	0.20
100	0.2	46	1.80	1.18	0.60	0.50
100	0	72	4.08	2.78	1.82	0.99
100	0.2	72	5.80	5.60	5.82	5.37
200	0	30	1.70	1.20	0	0
200	0.2	30	3.50	2.30	0.72	0.18
200	0	72	9.20	10.72	9.20	5.33
200	0.2	72	9.43	9.48	11.75	10.20

[3]These pH values were 5.5, 6.0, 6.5, and 7.0 before autoclaving. pH values given are for uninoculated tubes after autoclaving.

Medium of Henderson and Snell (20) except 2γ calcium pantothenate instead of 10γ and amino acids of Stokes et al (54) with threonine omitted.

the organism. However, at pH 6.7 and 7.05 the growth in response to 200 γ DL-serine is enhanced by the addition of 0.2 mg. Ca^{++}. This observation supports the hypothesis that the stability of alkaline earth complexes with citrates decreases as the pH is lowered. The decreased Ca^{++} requirement at lower pH also increases the validity of the assumption which postulates that the formation of lactic acid by the microorganism results in a release of Ca^{++} from the buffer complex. The importance of inhibition of growth by media constituents also becomes more significant in considering the pH effect. Any medium condition which prevents initiation of growth or attainment of maximum growth results in a slower decrease in pH and therefore a slower release of Ca^{++} from the buffer complexes.

Data in Table XXX also presents the growth initiation ability of Ca^{++} and a similar effect obtained by decreasing the pH of the medium. For example, titrations obtained at 30 hours with tubes containing 200 γ DL-serine show that early growths are obtained at pH 5.8 and 6.3, but not with media of higher pH. With the addition of Ca^{++} growth is extended to the higher pH levels and titrations at lower pH are increased.

From the evidence presented it can be concluded that Ca^{++} is required by L. delbrueckii. Although the absolute Ca^{++} requirement may be the same under all conditions tested, the apparent Ca^{++} requirement is determined by the buffer and other media conditions employed.

CHAPTER VII

SUMMARY

SUMMARY

1. Microbiological assay methods have been applied to the analysis of a flour prepared from the Chinese tallow nut and a protein preparation from the same source and inferences have been drawn as to the nutritional value of these materials for animals.

2. Both materials have a relatively high content of thiamin and pyridoxine but are relatively poor sources of riboflavin and pantothenic acid.

3. With respect to the majority of the nutritionally important amino acids the products from the tallow nut compare favorably with other foodstuffs which are considered adequate when fed at the level of 20% protein. However, the tallow nut products are deficient in both lysine and methionine and would not serve successfully as a sole source of protein for animals.

4. In the course of the microbiological investigations it was found that threonine inhibits the utilization of serine by L. casei, L. delbrueckii, Leuc. mesenteroides P-60, and S. faecalis R. Ratios of threonine to serine of 75, 150, 1500, and 2500, respectively, prevented the growth of these microorganisms. The importance of this observation in the assay of serine by the microbiological method is discussed.

5. The essential or non-essential nature of serine for S. faecalis R was found to be a function of the concentration of certain basal medium conditions. High levels of adenine, guanine, and uracil, low folic acid, and low amounts of Fe^{++} with a citrate buffered medium were necessary to establish serine as an essential metabolite for S. faecalis R. As the concentration of the purines and uracil was decreased and the concentrations of folic acid and Fe^{++} were increased, serine became non-essential for

S. faecalis R. These data, along with the observed toxicity of high levels of folic acid, suggested that serine was synthesized by an enzyme system which contained (1) a compound structurally related to adenine, (2) a compound similar to folic acid, and (3) Fe^{++} as an activator.

6. Calcium was demonstrated to be required for L. delbrueckii but the amount of Ca^{++} required by the microorganism varied with the organic acid buffer used. Inhibitions and lags in the initiation of growth also influenced the amount of Ca^{++} required in the medium. The influence of the buffers on the Ca^{++} requirement was explained on the relative abilities of the organic acids to form complexes with the alkaline earth cations. The role of inhibitions and lags in determining the Ca^{++} requirement of L. delbrueckii was approached on the basis of the decrease in stability of the complex between the alkaline earth cations and the organic buffers as the pH of the medium was lowered.

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